



# Effects of Heat Stress on Mating Behavior and Colony Development in *Bombus terrestris* (Hymenoptera: Apidae)

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### Specialty section:

This article was submitted to  
Ecophysiology,  
a section of the journal  
Frontiers in Ecology and Evolution

**Received:** 27 July 2021

**Accepted:** 30 September 2021

**Published:** 21 October 2021

### Citation:

Przybyla K, Michez D, Zambra E, Anselmo A, Hennebert E, Rasmont P and Martinet B (2021) Effects of Heat Stress on Mating Behavior and Colony Development in *Bombus terrestris* (Hymenoptera: Apidae). *Front. Ecol. Evol.* 9:748405. doi: 10.3389/fevo.2021.748405

Climate change is related to an increase in the frequency and intensity of extreme events such as heatwaves. In insect pollinators, heat exposure is associated with direct physiological perturbations, and in several species, could lead to a decrease of fitness related to a decrease in fertility. Here we developed a new experimental protocol in controlled conditions to assess if the exposure to high temperatures could modify the attractiveness and fertility of *Bombus terrestris* males. Our results show that virgin queens of *B. terrestris* do not have preferences between the pheromonal secretions of heat-exposed and control males. Moreover, mating with a heat-exposed male has no impact on the copulation behavior and the development of the nest (brood composition). We advise to extend trials to cover a range of wild and heat-sensitive species on multiple generations to better understand the impact of heat waves on the bumblebee communities.

**Keywords:** climate change, heat exposure, bumblebee, reproduction, colony development

## INTRODUCTION

Climate change is threatening worldwide biodiversity for many years in various direct (e.g., heat stress, drought) and indirect (e.g., habitat modification) ways (Bálint et al., 2011; Bellard et al., 2012; Dirzo et al., 2014). One of the consequences of exposure to heat stress is a loss of fertility (Walsh et al., 2019). In insects, heat exposure can impact the viability, motility, competitiveness, and the DNA integrity of spermatozoa (Sales et al., 2018; McAfee et al., 2020; Martinet et al., 2021b). With the increase in frequency and severity of heat waves (Meehl and Tebaldi, 2004; Hallmann et al., 2017) and hence the heat stress experienced by insects, this impact will worsen in the future and may cause negative population trends (Sales et al., 2018, 2021). We need to develop experimental protocols to assess and understand direct impact of heat stress on insect model species.

With an annual economic value of 153 billion dollars (USD) for crop pollination (Potts et al., 2016), insect pollinators, wild and domesticated, are a key functional group to

conserve (Williams, 1994). Recently, some species have been studied to assess the impact of environmental stresses (e.g., extreme temperatures, pesticides) on individual survival and fertility (McAfee et al., 2020; Minnameyer et al., 2021; Strobl et al., 2021), but the potential consequences of sublethal effects on resulting offspring have been poorly studied (Sales et al., 2018).

Here, we explore this question using bumblebees as a model species for eusocial pollinators. Bumblebees are particularly sensitive to environmental stressors, especially to extreme high temperatures as they are originally adapted to cold climate (Heinrich, 2005). Unlike most insects, bumblebees can individually regulate their body temperature to a certain species-specific point ( $CT_{min}$ ,  $CT_{max}$ ) regardless of the air temperature (Heinrich and Heinrich, 1983; Oyen et al., 2016). Moreover, temperature can also be regulated by workers at the colony level, around an optimal temperature of 30°C (Heinrich and Heinrich, 1983; Livesey et al., 2019). However, as emerged males live outside the nest, they cannot benefit from social thermoregulation. This exposure can lead to several physiological perturbations like a decrease in sperm viability and an increase in chromatin degradation (Martinet et al., 2021b). Degradation of sperm quality in bumblebee males can potentially be harmful because most studied bumblebee queens are monandrous (Baer, 2003). After storing sperm in their spermatheca for several months during the hibernation, queens use it to fertilize their eggs and produce diploid workers while unfertilized eggs produce males (Lecocq et al., 2017). If a queen mates with a heat-exposed male, it may receive a low-quality stock of spermatozoa. This could lead to a bias in favor of males in the sex ratio in the resulting colony and thus impact the reproductive efficiency of the colony (Beekman and van Stratum, 1998; also in solitary bees, Zaragoza-Trello et al., 2021).

To avoid mating with males with low fertility, females of bumblebees could be able to detect this change in fertility by an equally changed pheromone signal following heat stress. In bumblebees, the courtship behavior (e.g., patrolling) involves a release of pheromones known as Cephalic Labial Gland Secretions (CLGS; Calam, 1969; Ayasse and Jarau, 2014; Valterová et al., 2019). These species-specific secretions are intended to attract females before mating (Bergström et al., 1981). It has previously been shown that extreme heat temperatures could impact those secretions in heat sensitive bumblebee species (i.e., Martinet et al., 2021b).

In the present study, we present a new experimental approach to assess the impact of heat stress on the attractiveness, the mating behavior and the offspring of males of *Bombus terrestris* (Figure 1). As a model species, we used *Bombus terrestris*, a Euro-Mediterranean species domesticated for crop pollination (Rasmont et al., 2008). This species is highly tolerant to environmental changes and is expanding globally (Inari et al., 2005; Torretta et al., 2006; Rasmont et al., 2015; Martinet et al., 2021a). It has previously been shown that for this heat-tolerant species, no effect of heat exposure was observed on sperm viability, sperm DNA integrity and composition of the attractive secretions of males (Martinet et al., 2021b). However, other non-assessed parameters related to bumblebee reproduction (e.g., sperm mobility, attractiveness of pheromonal mixture) could

have a key role in the fertility of males. These factors are thus considered here.

## MATERIALS AND METHODS

### Model Species and Breeding Parameters

We used the buff-tailed bumblebee (*B. terrestris*) as a model species even though it is considered a resistant species (Martinet et al., 2021a) because it is easy to manage and available all year. Colonies were bought at Biobest NV (Westerlo, Belgium). We reared the different colonies in plastic boxes (8 cm × 16 cm × 16 cm) provided by Biobest NV (Westerlo, Belgium) at their optimal temperature of 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, Westerlo, Belgium) as sugar resources and pollen of *Salix* sp. as lipid/protein resources (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, with previous candies removed to prevent decay. Males were produced from queen-right colonies (colonies developing normally with a queen) and were isolated in plastic boxes without workers. To ensure that all 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 peak of pheromones production of mature males (Baer, 2003; Coppée et al., 2011; Ayasse and Jarau, 2014).

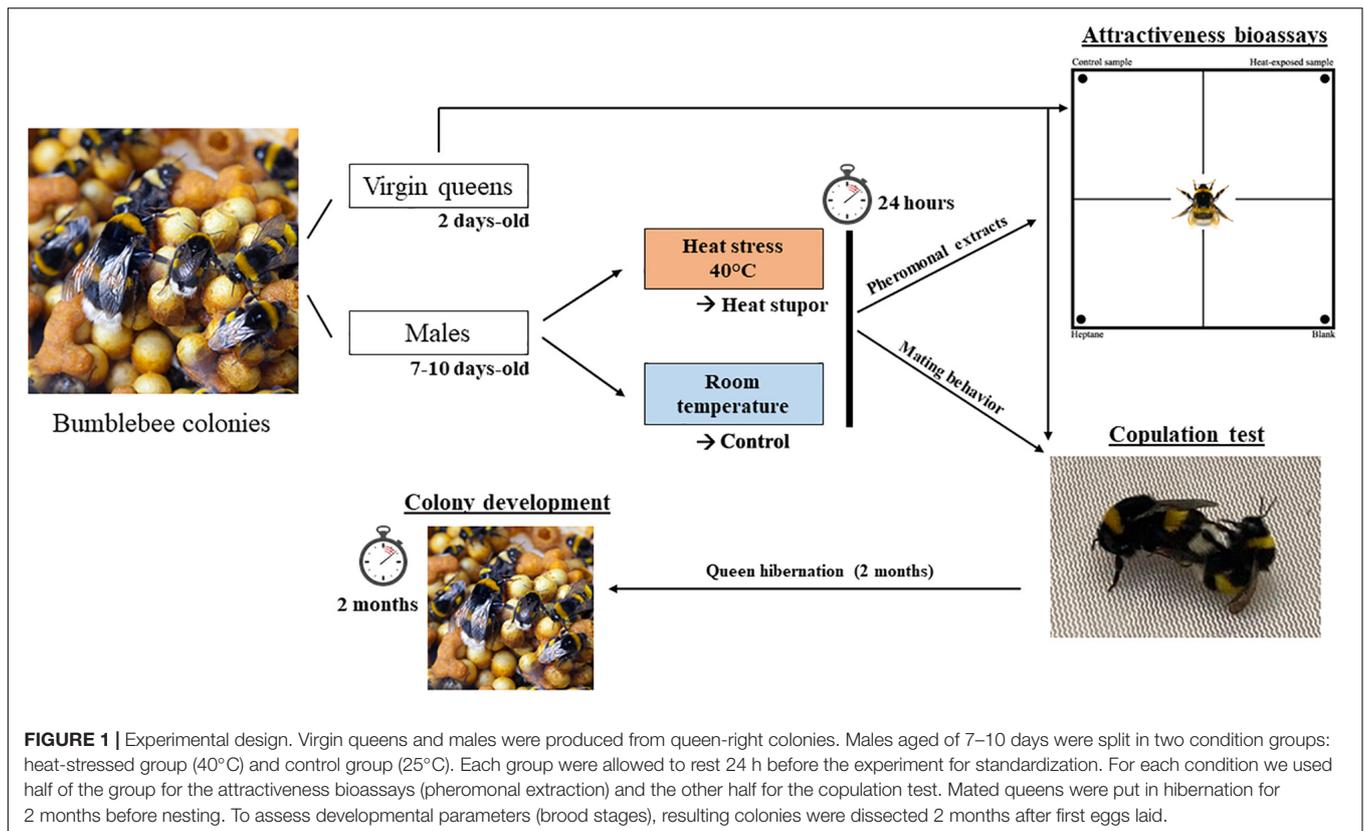
All queens and males were conditioned in standard plastic box (16 cm × 11 cm × 9 cm) with *ad libitum* pollen candies and sugar under controlled lab conditions (26°C and 50–60% of humidity). Queens were isolated in a standard plastic box (16 cm × 11 cm × 9 cm) with *ad libitum* pollen candies and sugar solution (Biogluc, Biobest, Waterloo, Belgium) 1 day before the mating experiment to improve the mating success (Röseler, 1985).

### Exposure of Males to Extreme Temperatures

Bumblebee males ( $n = 75$ ) were thermally exposed following the protocol of Martinet et al. (2015). Briefly, males were placed in an incubator at 40°C (typical heatwave temperature) until the “Heat Stupor” state. This state is recognizable by some behaviors: (i) the bumblebee falls upside down and is unable to turn back on its legs, (ii) it loses all its reflexes, and (iii) it has muscular spasms in its legs. After heat stress, survival males recovered 1 day in plastic boxes at 26°C with *ad libitum* access to nutritive resources (i.e., sugar) before being presented to virgin queen for the mating assays.

### Quality Check of Cephalic Labial Gland Secretions

A subset of the control and heat stressed males ( $n = 15$  and  $n = 15$ , respectively), were killed for pheromones quality

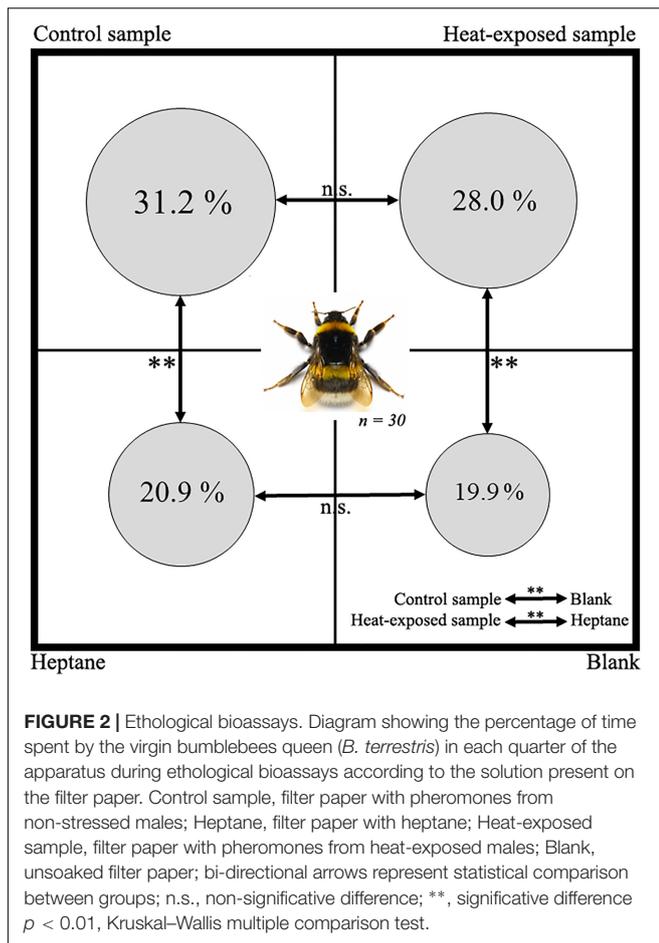


check. Cephalic labial gland secretions (CLGS) were extracted in 400 mL of heptane following a protocol adapted from De Meulemeester et al. (2011). We used gas chromatography-mass spectrometry using a Finnigan GCQ quadrupole system (GC/MS) with a non-polar DB-5 ms capillary column [5% phenyl (methyl) polysiloxane stationary phase; column length 30 m; inner diameter 0.25 mm; film thickness 0.25  $\mu\text{m}$ ] to determine the qualitative composition of CLGS. The identification was confirmed with analytical standards. We performed a relative quantification for 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  $\mu\text{m}$ ] and a flame ionization detector.

## Attraction of Virgin Females to Male Secretion

Virgin queens of *B. terrestris* are known to be very reactive to male pheromonal secretions in solution (Lecocq et al., 2015). We performed ethological tests based on the protocol established by Terzo and Sinkevich (2005) and updated by Lecocq et al. (2015). Virgin queens were stored individually in breeding conditions (i.e., 26°C, 50–60% of relative humidity and food resources *ad libitum*). To minimize potential external disturbances, ethological tests were carried out in the breeding room. All ethological tests were filmed using a camera (HC-100, Panasonic, Osaka, Japan) positioned at 81 cm above the

experimental device. Individuals were introduced one by one into the apparatus (70 cm  $\times$  70 cm  $\times$  8 cm). The device was divided into four square areas (35 cm  $\times$  35 cm), henceforth referred to as “quarters.” A plastic basket (85 mm  $\times$  85 mm  $\times$  24 mm, VWR International, Fontenay-sous-Bois, France) was placed in the corner of each quarter of the apparatus containing a piece of filter paper inside (1.5 cm  $\times$  1.5 cm) soaked with a 2  $\mu\text{l}$  sample of pheromonal secretions extract from control or heat-exposed males previously used for the CLGS quality check. Filter papers soaked in heptane (the solvent used for CLGS extraction) or not soaked were used as controls to test the attractiveness of the heptane itself. Therefore, the virgin queens were able to choose between four quarters during a same experiment: two quarters with either heptane or an unsoaked paper and the two other quarters with pheromonal secretions from either a control or heat-exposed male (Figure 1). Each young virgin queen was tested with a different sample from control or heat-exposed males randomly selected among the extracted pheromones of the 30 males. After each test, baskets and filter papers were removed, the apparatus was cleaned with ethanol and then ventilated for 5 min to ensure complete ethanol evaporation. Filter papers were placed randomly in the quarters to avoid a potential quarter preference factor. Each test was performed for a 5 min period under red-light conditions. The first 2 min correspond to the time required for virgin queens to be receptive to the new environment and were therefore removed from the analysis (Lecocq et al., 2015). During the last 3 min, we recorded the time spent by the queens in each quarter of the apparatus (we considered that the queen



was in a quarter when more than half of its body was in this quarter). Before experiment, neutrality tests ( $n = 30$ ) were carried out to check that no quarter was more attractive than others. Ethological bioassays were carried based on the CLGS of 30 males of *B. terrestris* (heat-exposed group  $n = 15$ ; control group  $n = 15$ ) and with 30 virgin queens, both from different colonies to avoid brother-sister mating.

## Copulation

Reproduction and hibernation of bumblebee queens was performed following Lhomme et al. (2013). For the reproduction experiment, we used 50 virgin queens (2-day old) provided by Biobest (Westerlo, Belgium). Each queen was placed in a flight cage (35 cm × 35 cm × 60 cm) exposed to natural light at room temperature with three different males (7–10 days old) of the same condition (control or heat-stressed) to stimulate the mating. To avoid genetic and behavioral bias, we used non-related males to virgin queens from different standardized colonies provided by Biobest (Westerlo, Belgium). Once a queen was mated with a male, the other males were removed from the device to avoid any disruption during the mating. After a successful mating ( $\pm 30$  min), the queen was placed in a plastic box (16 cm × 11 cm × 9 cm) during 1 week with *ad libitum* pollen and sugar before hibernation.

For the hibernation, bumblebee queens ( $n = 50$ ) were placed individually in a match box with a moist cotton to maintain humidity. Those boxes were maintained at 5°C for a period of 2 months (Beekman et al., 1998). After the hibernation, the queens were placed in a fly cage (Yoon et al., 2010) (30 cm × 30 cm × 30 cm) for 1 week at room temperature with *ad libitum* pollen and sugar. They were subjected to a photoperiod of 8 h of light and 16 h of dark for 3 days and then a phase of 24 h of full light to exit hibernation (Yoon et al., 2010). Finally, all the queens were placed individually in a rearing box (16 cm × 11 cm × 9 cm, Biobest NV, Westerlo, Belgium) to initiate their colony.

## Colony Development

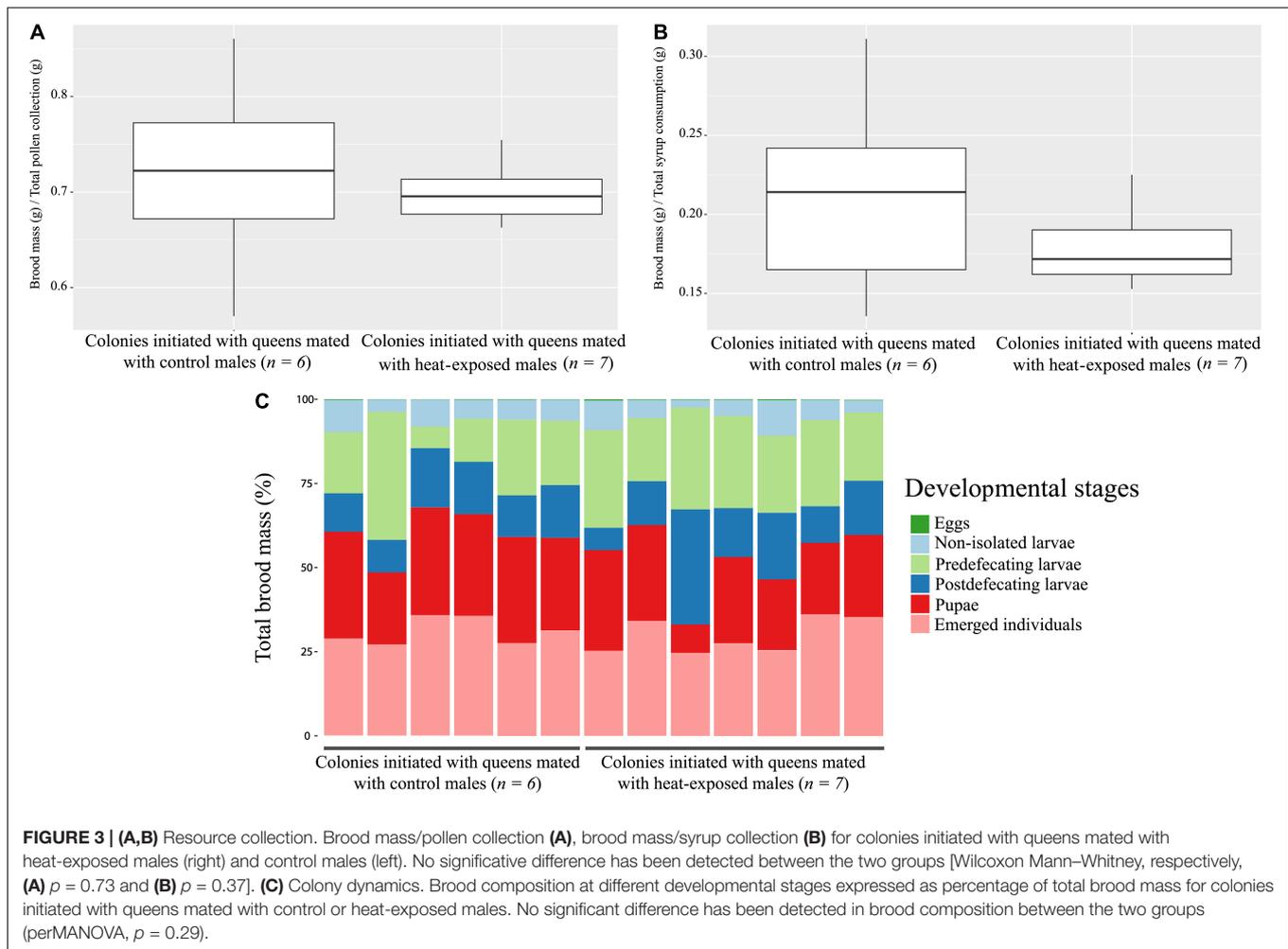
Queens were reared with optimum temperature conditions (26°C) and constant humidity (50–60%) in rearing plastic boxes (16 cm × 11 cm × 9 cm) in complete darkness. They were fed *ad libitum* with *Salix* sp. pollen (Ruchers de Lorraine), a diet of optimal quality (Vanderplanck et al., 2019) with sugar syrup (Biogluc, Biobest, Westerlo, Belgium). Prior to bumblebee feeding, pollen loads were crushed and mixed with sugar syrup (Biogluc, Biobest). Each pollen candy was weighed and replaced every 2 days and the removed candies were weighed to assess pollen consumption. During all the experimentations, syrup consumption was measured for each colony.

Colonies were dissected after 2 months of development (i.e., 2 months after the first eggs were laid). We selected this experiment time because it corresponds to a classic lifespan of commercial bumblebee colony and this time allows us to have a sufficiently grown colony to assess development parameters (Cnaani et al., 2002). We dissected the colonies using the following parameters defined by Tasei and Aupinel (2008): (a) total pollen and syrup consumption which gives a proxy on the larval development; (b) total mass of the brood; (c) number of new emergences (workers and possible reproductives) and (d) mortality (i.e., number of dead individuals divided by total individuals including initial ones and new-emerged ones) which gives indication on the workforce, brood care behavior and resilience (i.e., ability to respond to environmental stresses). We determined mass and number of each larval stages and emerged individuals (i.e., brood composition) considering separately the different brood stages, namely eggs, non-isolated larvae, isolated and pre-defecating larvae, isolated and post-defecating larvae, pupae, non-emerged and emerged males.

## Statistical Analyses

For ethological data, all the videos were analyzed by using the BORIS v.7.4.7 software (Friard and Gamba, 2016). The time spent in each quarter of the apparatus was analyzed by using a non-parametric Chi2 as the normality and homoscedasticity were not respected. A Kruskal–Wallis test was performed to test the difference between the different quarters followed by a *post hoc* Kruskal–Wallis multiple comparison test to identify which quarters are different from the others.

We performed statistical comparative analyses of the colony performances using R 3.5.1 environment (R Core Team, 2018). Significant tests (Wilcoxon Mann–Whitney) were run to assess



differences between groups for each parameter. For the analysis of brood composition (relative mass of the different larval stages), we performed a permutational multivariate analysis of variance (perMANOVA) on arcsine-transformed data using the Bray–Curtis dissimilarity matrix and 1000 permutations (“adonis” command, R-package *vegan*, Oksanen et al., 2018). Prior to these tests, the multivariate homogeneity of within-group covariance matrices was verified using the “betadisper” function. When a significant difference was detected, we performed multiple pairwise comparisons with an adjustment of  $p$ -values (Bonferroni correction). Finally, separate  $t$ -test and Tukey *post hoc* tests were conducted to assess the effect of heat exposure on each developmental stage.

## RESULTS

### Attraction of Virgin Females to Male Secretion

Quality check of CLGS revealed no difference among males and a sufficient load of pheromonal compounds including the main compound 2,3-dihydrofarnesol. Gas chromatography analyses

revealed a typical chemical fingerprint of *B. terrestris*. Our results confirm that the 7–10 days-old males can be used to assess the attractiveness of their sexual attractive mixture.

Neutrality tests performed before the experiments showed no significant differences between quarters of the apparatus (Kruskal–Wallis multiple comparison test,  $p = 0.31$ ), meaning that, without CLGS extract, young virgin queens have no preference for a specific quarter. During attractiveness tests, the time spent by the young virgin queens was not significantly different between the two control tests (i.e., performed with filter paper soaked in heptane or with unsoaked filter paper) (Kruskal–Wallis multiple comparison test,  $p = 0.19$ , **Figure 2**). However, a significant difference was detected between male secretions (from both control and heat-stressed groups) and heptane (Kruskal–Wallis multiple comparison test,  $p < 0.01$ ) and between male secretions and non-impregnated filter paper ( $p < 0.01$ ). Finally, no difference was detected between CLGS extracts from control and heat-exposed groups ( $p = 0.16$ ).

### Copulation and Colony Development

Overall, our results show that heat exposure on males did not have an impact on mating time or copulation behavior.

During nest development and colony growth, behavioral differences between both conditions (i.e., heat-exposed and control groups) as well as in colony dynamics and brood development were not observed. We evaluated the performance of bumblebee colonies based on colony growth, composition of brood, mortality, total pollen, and syrup collection. For both conditions (i.e., colonies from a heat-exposed male and colonies from a control male), all these parameters were not significantly affected by heat stress applied beforehand to the males (Kruskal–Wallis multiple comparison test, respectively,  $p = 0.67$ ;  $p = 0.89$ ;  $p = 0.73$  and  $p = 0.37$ ; **Figures 3A,B**). Finally, the dissection of the colony showed no significant difference in brood composition for all assessed developmental stages (**Figure 3C**, perMANOVA,  $p = 0.29$ ).

## DISCUSSION

In insects, heat stupor is associated to neuro-muscular potential, water balance, or physiological disruptions (Kingsolver et al., 2013). We performed multiple bioassays to investigate if heat-exposed males (i.e., in heat stupor with) are able to attract and mate with virgin queens and produce viable offspring. Our results demonstrate that virgin queens of *B. terrestris* are unable to distinguish secretions of heat-exposed males from secretions of non-exposed individuals. A previous study in another hymenopteran (i.e., *Nasonia vitripennis*, a parasitic wasp) also showed that females are not able to distinguish a heat-exposed male from a non-exposed male (Chirault et al., 2015). As previously demonstrated (Martinet et al., 2021b) it has been shown that there is no significant impact of heat shock on the histology of cephalic labial glands and the composition of the produced pheromones in males of *B. terrestris* (i.e., a warm-adapted species). Our bioassays are consistent with these results.

Regarding the experiment of copulation and colony development, our interpretations are limited to *B. terrestris*, a species known for its heat resistant capabilities. Additional experiments on more sensitive species still must be performed to test if virgin females are able to avoid copulation with heat-exposed males presenting potential physiological damages (Martinet et al., 2021a,b). Indeed, it has been emphasized that bumblebee species, like the declining *Bombus jonellus*, are more substantially impacted by heat exposure particularly in CLGS composition, sperm viability and sperm DNA integrity (Martinet et al., 2021b). In the present study, we investigated the effect of heat stress on the first generation only, although it is known that extreme high temperatures could cause a decrease in sperm viability and thereby transgenerational damages on different life stages in ectotherm insects, as it is the case for the red flour beetle *Tribolium castaneum* (Sales et al., 2018, 2021). However, while investigating transgenerational damages could be a key point to understand global pollinator decline, we did not detect any significant impact in *B. terrestris* while in the case of *Tribolium castaneum*, damages

from thermal stress is observable in the first generation (Sales et al., 2018, 2021).

Different threats have been identified to explain the global decline of bumblebees. For several years, many studies have explored effects of microbiota, diet, temperature, and pesticide exposure on bumblebee's health in lab conditions (Gill and Raine, 2014; Vanderplanck et al., 2019; Barraud et al., 2020; Rothman et al., 2020; Maebe et al., 2021a; Oyen et al., 2021). However, most of these studies used commercially available bumblebee species (i.e., *Bombus impatiens* in North America and *B. terrestris* in Europe, and *Bombus hypocrita* in Asia) which are known to be tolerant and resistant to environmental changes (Oyen and Dillon, 2018; Maebe et al., 2021b; Martinet et al., 2021a). Limiting studies to reared species may bias our overall view of the adaptive response of bumblebees to decline factors as these species are selected throughout this rearing process. We should extend further studies to more wild and sensitive species to better understand this impact on the global bumblebee's biodiversity.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

KP, EZ, AA, and BM conceived and designed the experiments, and performed the experiments. KP and BM analyzed the data. All authors wrote the manuscript.

## FUNDING

KP is Ph.D. student “Aspirant” of F.R.S.-FNRS and BM is F.R.S.-FNRS grant fellowship “Chargé de Recherches” (Fonds de la Recherche Scientifique). DM contributed as researcher for the “Fonds de la Recherche Scientifique – FNRS,” the Research Foundation – Flanders (FWO) under EOS Project named CLIPS (n°3094785). The research was partly funded by the Institute of Biosciences (UMons) under “Bombstress project”.

## ACKNOWLEDGMENTS

We acknowledge all people who helped us to carry out this experiment, especially Dimitri Evrard and Victor Lefebvre for their technical support and the maintenance of bumblebee breeding room. We thank Maryse Vanderplanck for her advice and discussions during the experimentations and Thomas J. Wood for the proofreading. We also thank the two reviewers for their constructive comments which have greatly improved the quality of the manuscript.

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