

RESEARCH ARTICLE

Experimental short-term heatwaves negatively impact body weight gain and survival during larval development in *Bombus terrestris* L. (Hymenoptera: Apidae)

Laura Wögler and Christoph Kurze*

ABSTRACT

Climate change-induced heatwaves threaten global biodiversity, including crucial pollinators like bumblebees. In particular, the increasing frequency, duration and intensity of heatwaves is alarming. Despite these projections, little is known about the effects of short-term heatwaves on insect larval development. Hence, we investigated the impact of simulated heatwaves on the development of 4th instar larvae (L4) of *Bombus terrestris* L. (Hymenoptera: Apidae) using an *in vitro* rearing method. Individual larvae were incubated at 37°C and 38°C for a period of 4 days, with a constant rearing temperature of 34°C as the control. We examined body weight gain, developmental duration, survival to adult stage, and adult body size (i.e. dry mass, intertegular distance, and head width). A simulated heatwave of 37°C did not significantly affect larval development, but 38°C impaired larval body mass gain. While developmental duration and adult body size were unaffected, an acute heat stress of 38°C during the L4 stage reduced the probability of pupae reaching adulthood. These findings highlight the potential for heatwaves to negatively affect bee populations by impairing larval growth and reducing survival to the adult stage, which may have severe implications for colony fitness.

KEY WORDS: Social insect, Extreme heat, Acute stress, Critical weight, Metamorphosis, Brood, *Bombus terrestris*

INTRODUCTION

Climate change is a major challenge of the 21st century, causing cascading effects that impact weather patterns, biodiversity, and entire ecosystems (Garcia et al., 2014; IPCC, 2023). Particularly concerning are heatwaves, defined as at least three consecutive days with extreme heat, which have increased in frequency and intensity (Lhotka et al., 2018; Perkins-Kirkpatrick and Lewis, 2020; Stillman, 2019). These heatwaves have a significant impact on terrestrial animals, including humans (Stillman, 2019), small mammals (Fuller et al., 2021; Ratnayake et al., 2019; Zhao et al., 2020), birds (Conradie et al., 2019; McKechnie and Wolf, 2010), and insects (Bodlah et al., 2023; González-Tokman et al., 2020; Ma et al., 2021). Despite this threat, our understanding of the impact of heatwaves on animal

development, their fitness, and populations remains limited (Fuller et al., 2021; González-Tokman et al., 2020; Jentsch et al., 2007; Ma et al., 2021; Stillman, 2019).

Cold-adapted heterothermic species like bumblebees (*Bombus* sp.), which have species-specific distributions ranging from the Arctic to temperate/Mediterranean regions, may be particularly at risk (Ghisbain et al., 2024; Maebe et al., 2021; Martinet et al., 2021a; Rasmont and Iserbyt, 2012; Soroye et al., 2020; Suzuki-Ohno et al., 2020). As key pollinators in many ecosystems and for agriculture (Cameron and Sadd, 2020; Corbet et al., 1991; Klein et al., 2007; Potts et al., 2016), understanding how extreme heat events impact their physiology and fitness is crucial. Although heat tolerance varies greatly between species, with alpine and polar species being particularly sensitive to heat stress (Martinet et al., 2021a; Zambra et al., 2020), commercially used bumblebees such as *Bombus terrestris* are typically housed aboveground, making them more frequently exposed to extreme heat. Recent studies show that heat stress can reduce adult survival (Kuo et al., 2023; Quinlan et al., 2023) and elevated temperatures may lower colony fitness (Martinet et al., 2021b; Theodorou et al., 2022). Under heatwave-like temperatures, adult bumblebees exhibit impaired cognition (Gérard et al., 2022a) and scent perception (Nooten et al., 2024), and display altered fanning and foraging behaviours (Bretzloff et al., 2024; Kuo et al., 2023; Sepúlveda et al., 2024), potentially affecting colony fitness. Interestingly, foraging behaviour and response to stimuli are altered in adults even when exposed to heatwave-like temperatures during their larval and pupal development (Gérard et al., 2022b; Perl et al., 2022). Exposing entire colonies to high temperatures for extended periods can lead to alterations in wing size asymmetry, wing shape and size, and reductions in body and antennae sizes (Guiraud et al., 2021; Gérard et al., 2022b, 2023, 2018; Perl et al., 2022). Despite that different developmental stages are likely to be variably affected, it is unclear which stages are particularly vulnerable to heatwaves and whether shorter extreme heat events are sufficient to impair their development.

To address this knowledge gap, we adapted an *in vitro* rearing protocol (Kato et al., 2022) to examine the direct impact of a 4-day-long heat stress period during the development of 4th instar larvae (L4). This experimental approach prevented heat mitigation through worker fanning activities (Weidenmüller et al., 2002). While *in vitro* rearing is a standard procedure in honeybee research (Crailsheim et al., 2013; Schmehl et al., 2016), it is rarely used in bumblebee research (Kato et al., 2022; Pereboom et al., 2003). Therefore, we chose *B. terrestris* as a model species, although it is known to be rather heat-tolerant (Martinet et al., 2021a; Zambra et al., 2020). We used this method to investigate changes in body mass, developmental duration, survival until pupation and emergence, and adult body size following a 4-day-long heatwave-like exposure during L4 development. Additionally, we assessed lipid content; as the fat body

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in adult bees is linked to reproductive success, immunity, stress resilience, and foraging efficiency, making it a potentially valuable marker for monitoring bee health (Vanderplanck et al., 2021).

RESULTS

A 4-day-long heatwave at the beginning of L4 development significantly impacted *B. terrestris*' probability of reaching adulthood ($\chi^2=6.48$, d.f.=2, $P=0.039$; Fig. 1C). Larvae exposed to 38°C were 50% less likely to reach adulthood compared to larvae exposed to 37°C ($P=0.024$) or the control group ($P=0.024$). However, there was no significant difference in survival between the 37°C group and the control group ($P=1$). The number of L4 larvae reaching the pupal stage was not significantly affected ($\chi^2=1.36$, d.f.=2, $P=0.507$; Fig. 1A). There was also no significant effect of heatwave treatment on the developmental times for larvae reaching pupal stage [generalized linear mixed effect model (GLMM): $\chi^2=2.50$, d.f.=2, $P=0.287$; Fig. 1B] and for pupae until emergence (GLMM: $\chi^2=0.54$, d.f.=2, $P=0.762$; Fig. 1D).

Heatwaves significantly affected the relative weight gain in L4 larvae during the treatment (glmm: $\chi^2=7.94$, d.f.=2, $P=0.019$; Fig. 2A). While exposure to 38°C revealed significantly lower weight gains compared to the control (Tukey HSD: t-ratio=2.80, $P=0.015$), there was no significant effect for larvae exposed to 37°C (t-ratio=1.61, $P=0.242$). Regardless of treatment, their body weight loss during pupation had no significant effect ($\chi^2=0.60$, d.f.=2, $P=0.741$; Fig. 2B). There were also no significant effects on morphometrics in 2-day-old adults, including their dry mass ($\chi^2=2.26$, d.f.=2, $P=0.322$; Fig. 2C), ITD ($\chi^2=2.58$, d.f.=2, $P=0.275$; Fig. 2D), head width ($\chi^2=2.25$, d.f.=2, $P=0.325$; Fig. 2E), and relative lipid content ($\chi^2=4.07$, d.f.=2, $P=0.131$; Fig. 2F). However, it is worth noting that the sample size was significantly reduced in the 38°C treatment group at the adult stage ($n=16$, see Fig. 1C,D).

We found that both the heatwave treatment and relative body weight gain during treatment impacted the probability of larvae reaching adulthood (treatment: $\chi^2=14.759$, d.f.=2, $P=0.0006$; weight gain: $\chi^2=24.35$, d.f.=1, $P<0.0001$), but not their

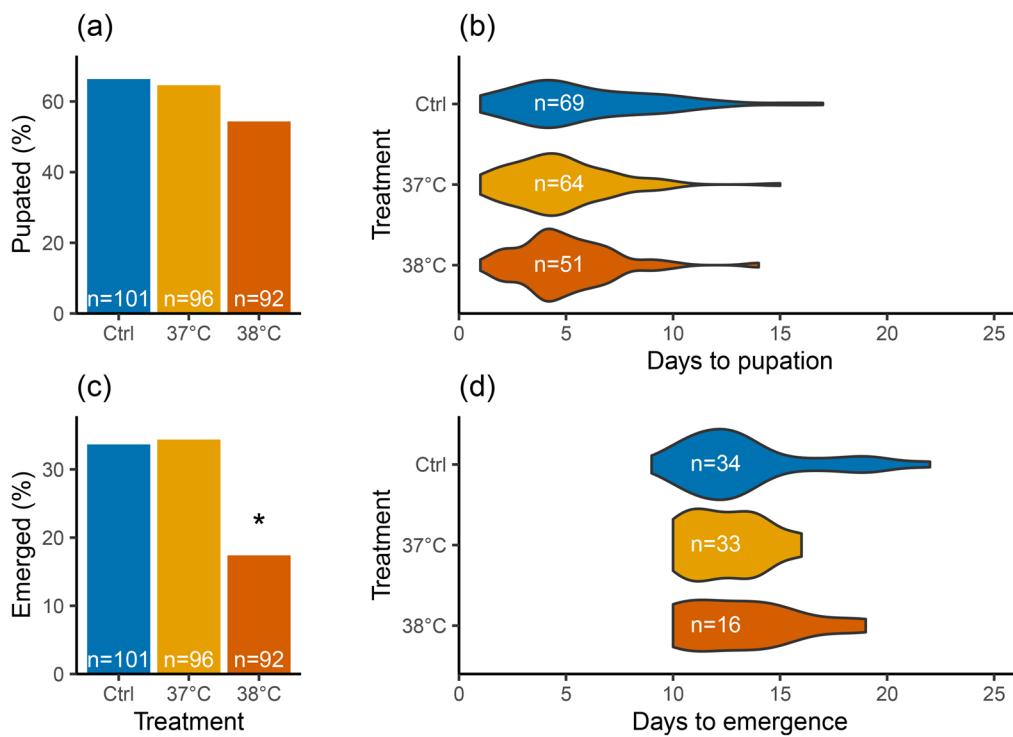
interactions ($\chi^2=0.04$, d.f.=2, $P=0.980$). In addition, we found that relative body weight gain had a significant effect on the probability of larvae to pupate ($\chi^2=14.10$, d.f.=1, $P=0.0002$).

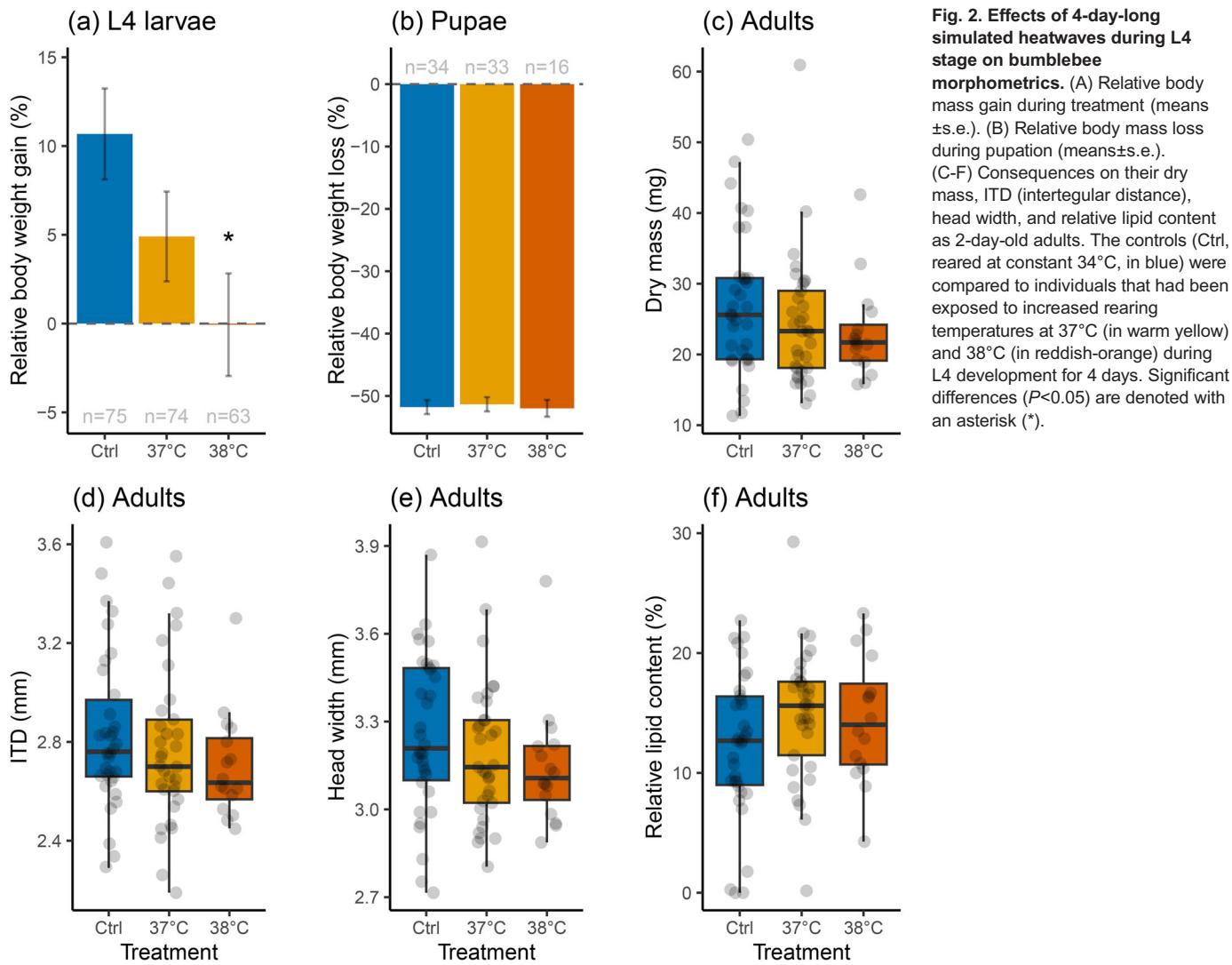
DISCUSSION

Our data provide evidence of how simulated short-term heatwaves during the L4 stage affect development and survival until adulthood in *B. terrestris* (Figs 1A and 2A). Nonetheless, there was no effect on the duration of larval development (Fig. 1B,D), nor on the morphometrics in adults (Fig. 2C-E). This contrasts with previous studies showing that exposing colonies to elevated temperatures produced smaller workers, as indicated by smaller ITD (Guiraud et al., 2021; Gérard et al., 2023), with reduced antennae in *B. terrestris* (Gérard et al., 2023). In addition, while wing size and shape can be affected (Gérard et al., 2018), this may not always be the case (Gérard et al., 2023). As only 17% of individuals emerged as adults in the 38°C treatment group of our experiment, this sample size was too small to thoroughly analyse wing morphology. Regardless, a potential explanation for this discrepancy could be that colonies were exposed to higher temperatures for extended periods in those experiments, whereas we tested the effect of short-term heatwave-like exposures in L4 larvae. This explanation is supported by another study showing that body or organ sizes were also not altered when colonies were exposed to elevated temperatures for shorter periods (Perl et al., 2022).

We found there was a 50% lower probability that L4 larvae exposed to 38°C would emerge as adults compared to both the 37°C heatwave group and the control group (Fig. 1C). Although the emergence rate in our *in vitro* rearing was low, our pupation rate, ranging between 55–66% irrespective of treatment, was similar to previous research (Kato et al., 2022). This suggests that 38°C might be a threshold temperature with ripple effects on critical processes during pupation. While insects typically exhibit left-skewed temperature responses, characterized by a steep decline in fitness beyond the temperature optimum, this pattern is generally more pronounced in tropical- rather than cold-adapted insects (Deutsch et al., 2008).

Fig. 1. Effects of 4-day-long simulated heatwaves on survival and developmental duration of L4 larvae (A,C) and pupae (B,D). The controls (Ctrl, reared at constant 34°C, in blue) were compared to individuals that had been exposed to increased rearing temperatures at 37°C (in warm yellow) and 38°C (in reddish-orange) during L4 development for 4 days. Significant differences ($P<0.05$) are denoted with an asterisk (*).





The evolution of complex thermoregulatory behaviours, including fanning, metabolic heat generation, and direct incubation of brood, likely resulted in a narrower optimal temperature range in bumblebee larvae, similar to that of honeybees (Jones and Oldroyd, 2006). Nonetheless, nest thermoregulation comes with increased energetic costs (Bretzlaff et al., 2023), which likely lead to reduced colony growth (Theodorou et al., 2022; Vogt, 1986). Above a certain temperature threshold, or when colonies are not large enough, maintaining nest thermoregulation may become unsustainable. For example, in *B. impatiens*, offspring production decreases at 35°C as workers abandon their colony (Bretzlaff et al., 2024). In *B. terrestris*, this threshold appears to be higher, with drone production increasing with elevated nest temperatures up to 34–36°C in microcolonies, at which point workers massively increase fanning activity (Sepúlveda et al., 2024). Besides differences between bumblebee species and populations (Zambrano et al., 2020), resilience to heat stress likely also differs between aboveground and belowground nesting species (Gonzalez et al., 2024). To limit confounding effects, such as worker behaviour, food supply and quality, we removed larvae from their natural nest environment and reared them *in vitro* under highly controlled conditions. While our experiment approach does not reflect a realistic scenario, *in vitro* rearing allowed us to control the timing and duration of heatwaves. Furthermore, it allowed us to

closely monitor weight gain and loss throughout larval development until emergence (Fig. 2A,B).

A 4-day-long exposure to 38°C during L4 development resulted in lower weight gain compared to both the 37°C heatwave and control groups (Fig. 2A). This suggests that larvae either consumed less food or experienced increased energetic costs that could not be compensated by food intake. While it is known that stress reduces food intake and consequently weight gain in mammals (Rabasa and Dickson, 2016), surprisingly little is known about how acute stress affects food consumption and body weight gain in insects. Reaching a critical weight during the larval development, however, is crucial for initiating molting and metamorphosis, as shown in the tobacco hornworm (*Manduca sexta* L., Lepidoptera: Sphingidae) (Nijhout and Williams, 1974). It has also been shown that reaching a critical weight can be influenced by both temperature and food quality (Davidowitz et al., 2003). Our data confirms this for bumblebees, with weight gain at the L4 stage being a significant predictor of pupation success, irrespective of the treatment group.

In 3rd instar fruit fly larvae (*Drosophila melanogaster* M., Diptera: Drosophilidae) short-term heat stress reduced food intake in adults on the day after emergence, without impacting their body weight but leading to increased glucose and trehalose levels while reducing lipid stores (Karpova et al., 2024). Although we did not

detect any significant difference in body size (i.e. dry mass, ITD, and head width) or relative lipid content in adult bees between treatment groups, we observed a notable decrease in body size variance with increasing heat stress (Fig. 2C-F). One potential explanation is that our extreme heat stress may have selected for individuals with adaptive traits, resulting in a more homogenous size distribution among the surviving bees. Another possibility is that the massive weight loss during metamorphosis may mask more subtle effects on adult body size. A third explanation could be the reduced sample size, particularly in the 38°C heatwave group, and the slightly higher number of males in the control group.

Although both the heatwave treatment and the weight gain had a significant effect on the probability of larvae reaching adulthood, there was no interaction between both factors. This suggests that weight gain similarly increased survival, regardless of the treatment group. Nevertheless, our data supports the hypothesis that acute stress during larval development has a drastic impact at a later life stage (Karpova et al., 2024), although effects could have also just been delayed in our experiment. An exposure to 38°C at the L4 stage marked a threshold at which processes during metamorphosis are likely impaired in *B. terrestris*. It would be interesting to see how acute stress in early development would impact the life-history of adult bees. It has already been shown that heatwave-like temperatures during late development impact initial behavioural responses to sensory stimuli in adult workers of *B. terrestris* (Perl et al., 2022). This could not only have detrimental effects for the individual worker but also ripple effects on colony fitness.

In conclusion, while our *in-vitro* rearing experiment showed a certain resilience of *B. terrestris* larvae to heatwave-like exposures up to 37°C, extreme temperatures of 38°C appeared to be the threshold where pupal development was severely impaired. Individuals reaching adulthood, however, did not differ in their body size (i.e. dry mass, ITD, and head width) and relative lipid content, suggesting potential adaptive advantages in those surviving bees. With our experimental approach, we aimed to investigate the specific effects of acute thermal stress at the L4 stage, which we traded for experimental realism. Larvae were taken out of their natural nest environment, which likely has a large impact on their survival. Although our selected temperatures may appear extreme, they are only 3°C and 4°C above what are considered to be optimal rearing conditions. Our heatwave scenarios closely reflect realistic conditions in commercially used bumblebees, which are typically housed aboveground. Since we studied the relatively heat-tolerant *B. terrestris* as a model species, we would expect more severe effects in cold-adapted species, such as *B. lapidarius*, *B. alpinus* or *B. polaris* (Martinet et al., 2021a). Additionally, the impact of heatwave could also be more pronounced under natural conditions, where colonies are exposed to multiple stressors simultaneously (Theodorou et al., 2022). Given the increasing frequency and severity of heatwaves, it

is crucial to investigate their impact on the life-history and adaptive potential of keystone species like bumblebees.

MATERIALS AND METHODS

Experimental overview

To simulate the effect of short-term heatwaves on larval development, we collected L4 larvae (a total of 289) from five commercial *B. terrestris* colonies to rear them *in vitro*. These larvae were pseudo-randomly assigned to one of the three experimental groups to ensure equal distribution among treatments and colonies. Larvae of the simulated heatwave treatment were exposed to either 37°C ($n=96$) or 38°C ($n=92$) for 4 days, while the control group (ctrl, $n=101$) was reared at a constant 34°C. The control temperature was chosen based on previous research (Kato et al., 2022), although it is at the upper range of the typical brood temperatures reported in earlier studies (Vogt, 1986; Weidenmüller et al., 2002). Our treatment temperatures aligned with our separate study on pupal development (Laußer and Kurze, 2025 preprint), aimed to simulate potential extreme heatwave scenarios in Europe. For example, heatwaves reaching or exceeding temperatures of 38°C have occurred in parts of the British Isles, Mediterranean and Eastern Europe in recent years (Barriopedro et al., 2011; Lhotka and Kyselý, 2024; Rita et al., 2020). Moreover, our treatments are still comparable to previous studies, considering that the actual brood temperature is typically 2°C warmer than the ambient nest temperature (Vogt, 1986; Weidenmüller et al., 2002). At these elevated treatment temperatures, *B. terrestris* workers spend significantly more time cooling the brood through fanning (Sepúlveda et al., 2024), suggesting that such temperatures could have adverse effects on larval development. We recorded body mass changes during treatment and pupal stage. Their survival was checked daily until adult bees emerged and reached the age of 2 days. These adult bees were freeze-killed and kept at -20°C for subsequent morphometric measurements and analysis of their dry mass and lipid content.

Colony husbandry

Upon arrival, each *B. terrestris* colony (Natupol Research Hives, Koppert B.V., Netherlands) consisted of 20-30 workers, brood, and one queen. They were housed and maintained under similar conditions as described previously (Gilgenreiner and Kurze, 2024). Briefly, bumblebees had access to 70% (w/v) sucrose solution *ad libitum* in a foraging arena [59 (l)×39 (w)×26 (h) cm] with 14:10 h light:dark regime. Depending on colony size, each colony received 6-11 g of pollen candy daily, consisting of 67% organic pollen (naturwaren-niederrhein GmbH, Germany), 25% sucrose and 8% tap water. The room temperature was maintained at 25°C±1°C and 30-50% relative humidity. We allowed all colonies to develop for at least 1 month before starting to collect L4 larvae for the experiment.

Collection of 4th instar larvae

In this study, we exclusively focused on 4th instar larvae because they are easily identifiable inside the nest by their individual, spherical brood cells with a small opening for food provisioning (Tian and Hines, 2018). Additionally, our pilot study showed that they can be reared *in vitro* more successfully than earlier larval stages without unacceptably high mortality rates. Before collecting L4 larvae for our experiment, we carefully removed all existing L4 larvae from each colony using soft tweezers. This allowed us



Fig. 3. *In vitro* rearing of L4 larvae.
 (A) Design of 3D-printed polylactide (PLA) artificial brood cells (capacity: 0.6 ml). (B) Artificial brood cells with L4 larvae inside a 24-well clear flat bottom plate, and (C) L4 feeding on medium.

to identify and collect larvae that just had entered the L4 stage during our daily colonies checks over the following days. To facilitate the collection process, we temporarily moved all adult bumblebees to a separate cage and returned them afterwards.

***In vitro* rearing**

We followed established *in vitro* rearing procedures with slight modifications (Crailsheim et al., 2013; Kato et al., 2022), where we carefully transferred L4 larvae into 3D-printed polylactide (PLA) artificial brood cells (capacity=0.6 ml, diameter=8 mm, **Fig. 3A**). This facilitated measuring their weight gain without touching them again. To simulate short-term heatwaves, we randomly assigned L4 larvae to one of the three experimental groups and either reared them at 37°C and 38°C for 4 days (KB115, BINDER GmbH, Germany). The control group (ctrl) was maintained at constant temperature of 34°C (Kato et al., 2022; Pereboom et al., 2003). Those artificial brood cells were placed into 24- or 48-well clear flat bottom plates (Falcon/Corning, USA) (**Fig. 3B**) and kept inside in ventilated plastic containers (18.5×18.5×11.5 cm) together with a 120 ml cup of saturated sodium chloride solution to maintain 65±10% relative humidity.

Larvae were fed with a pollen medium twice daily, in the morning and in the evening. The medium contained 50% w/v sucrose solution (Südzucker AG, Germany), 40% honeybee collected organic pollen (Bio-Blütenpollen, naturwaren-niederrhein GmbH, Germany), 10% Bacto yeast extract (Bacto™, BD, USA), and 1% casein sodium salt from bovine milk (Sigma-Aldrich, Germany). Aliquots of medium were stored at -20°C and warmed up to 34°C and vortexed before feeding. A feeding session consisted of two 20-min rounds on a heated plate at 35°C (Medax model 12801, Medax Nagel GmbH, Germany). Larvae were initially fed a 6 µl droplet (7.1±1.6 mg) of medium onto their ventral abdomen (**Fig. 3C**). We monitored larval behaviour to determine satiation. A larva was considered satiated when it curled up and ceased movement and hungry when it remained active (**Movie 1**). Larvae that did not consume food during the first feeding round were not offered additional food. At the end of the feeding session, any remaining food was carefully removed to prevent the larvae from suffocating due to dried food blocking their trachea. Larvae entering pupal stage were no longer fed. Due to the difficulty in accurately monitoring survival during the pupal stage, daily survival data were not included. Instead, we present the proportion of larvae that pupated and successfully reached adulthood.

Measurements of body mass

Each individual bee was weight at four different stages: as L4 larvae pre- and post-treatment, at the beginning of pupal stage, and as a newly emerged adult using a fine scale (d=0.1 mg, Sartorius AC120S, Sartorius AG, Germany). To reduce stress and avoid any potential handling damage of the larvae, we kept them inside their 3D-printed cell. Consequently, we subtracted the empty cell weight to obtain the actual weights.

Morphometric measurements, dry mass, and lipid content in adults

The intertegular distance (ITD) and dry mass serve as a proxy for adult body size (Gilgenreiner and Kurze, 2024; Kendall et al., 2019). In addition to the ITD, we measured the head width and determined the sex by counting antennal segments (females have 12, males 13) using a digital microscope (CHX-500F, Keyence GmbH, Germany). In total, 64 females and 19 males emerged as adults. We obtained individual dry mass and lipid content following our previous protocol (Gilgenreiner and Kurze, 2024). Briefly, we dissected the ventral abdominal segments and dried corpses at 60°C for 3 days in a drying cabinet (U40, Memmert GmbH & Co. KG, Germany). After weighing their dry mass (d=0.1 mg, analytic balance M-Pact AX224, Sartorius GmbH, Germany), we extracted their body lipid with petroleum ether for 5 days. After discarding the ether and rinsing them with fresh ether, the bees were dried for an additional 3 days and weighed again. The lipid content was calculated as the difference between the initial and post-extraction dry weights.

Statistical analyses

All statistical analyses and data visualizations were performed using R Statistical Software (v4.4.1; R Core Team, 2024). The complete code and

output are provided in the electronic [supplementary information](#). Briefly, the probability of L4 larvae reaching pupal stage and adulthood was calculated using *chisq-test* function to perform a Pearson's χ^2 test for count data with Yate's continuity correction. Pairwise comparisons of survival probabilities between survival treatment groups were conducted using the *pairwise.prop.test* function with Benjamini-Hochberg correction. In addition, we ran GLMMs using the *glmmTMB* package (Brooks et al., 2017) with either gaussian or gamma data distribution to analyse effects of the heatwave treatment as a fixed factor on the duration to pupation and to emergence, the relative body weight gain during treatment, the relative body weight loss during pupation, adult body size (with dry mass, ITD, and head width serving as proxies), and the relative lipid content as response variables. In addition, we ran another GLMM based on binomial data distribution to test whether both heatwave and relative body weight gain as well as their interactions affected their probability to reach pupal stage and adulthood. We included 'colony ID' as a random effect in all models to account for colony-specific variability. Additionally, we included 'sex' as a random effect in models involving adult body size measurements (dry mass and head width) and relative lipid contents to account for potential morphometric differences between workers and male bumblebees. Model selection was performed based on the Akaike information criterion (AIC) and likelihood ratio tests. The final models were compared with their respective null-models. Model assumptions and dispersion of the data were checked using the *DHARMA* package (Hartig, 2020). Significance levels ($P<0.05$) were determined using the *Anova* function of the *car* package (Zuur et al., 2009). Pairwise comparisons between treatment groups were conducted using the function *emmeans* (Lenth and Lenth, 2018) adjusted with Tukey's HSD.

Ethics statement

This study was conducted in accordance with the ethical regulations of the German Animal Welfare Act (TierSchG) for conducting experiments with insects.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: C.K.; Data curation: L.W., C.K.; Formal analysis: C.K.; Investigation: L.W., C.K.; Methodology: C.K.; Project administration: C.K.; Resources: C.K.; Supervision: C.K.; Visualization: C.K.; Writing – original draft: C.K.; Writing – review & editing: C.K.

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Data and resource availability

All relevant data can be found within the article and its [supplementary information](#).

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