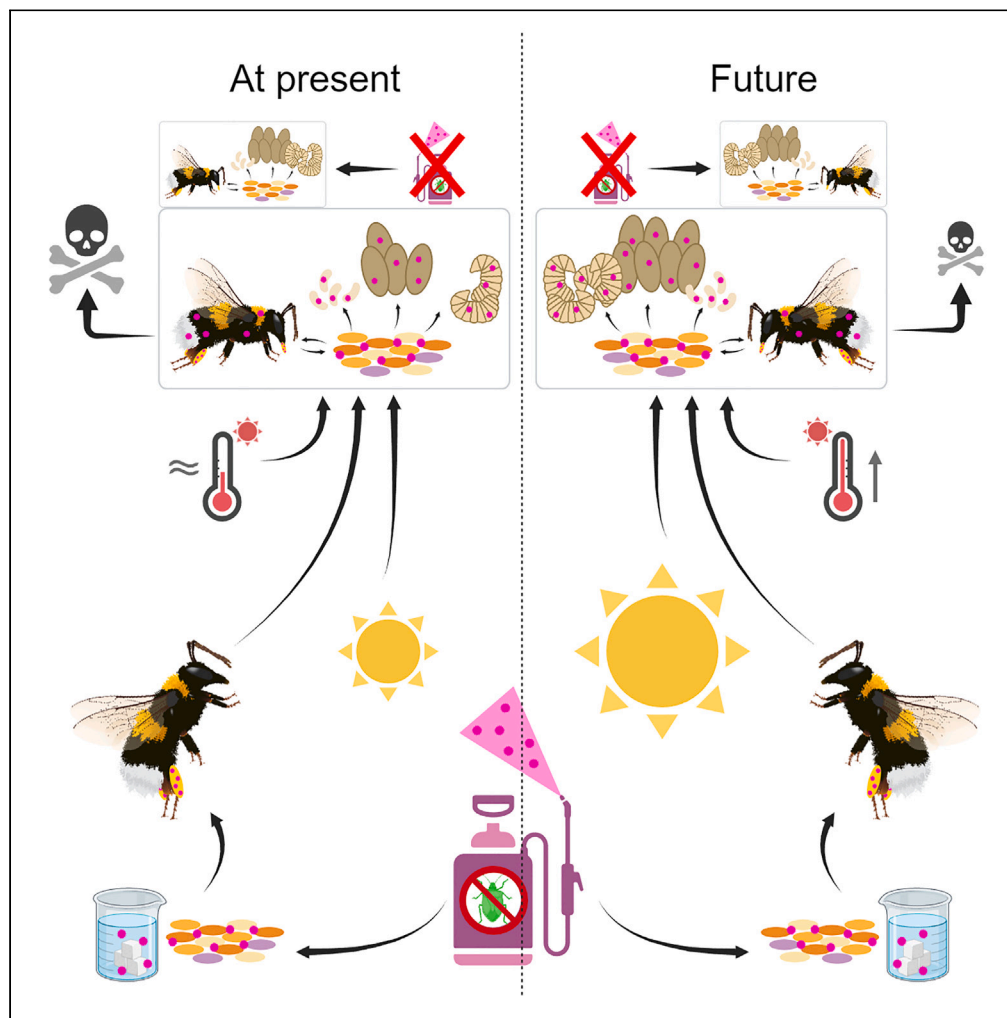


Article

Bumblebees under stress: Interacting effects of pesticides and heatwaves on colony development and longevity



Carmen A. Nebauer, Paula Prucker, Fabian A. Ruedenauer, Johannes Kollmann, Sara D. Leonhardt

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Highlights

Sulfoxaflor-treated pollen was consumed the least

Strongest negative effect with sulfoxaflor (survival, reproduction)

Colonies performed overall better in the future (warmer) heatwave scenario

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Article

Bumblebees under stress: Interacting effects of pesticides and heatwaves on colony development and longevity

Carmen A. Nebauer,¹ Paula Prucker,¹ Fabian A. Ruedenauer,¹ Johannes Kollmann,² and Sara D. Leonhardt^{1,3,*}

SUMMARY

Pollinator decline is linked to intensified agricultural practices, pathogens, climate change, and several other factors. We investigated the combined impact of heat and pesticide stress on food consumption, survival, and reproductive fitness of bumble bees. As climate change is expected to intensify heatwaves, we simulated a present-day and a future heatwave scenario (as expected in 50 years). In both scenarios, we exposed microcolonies to three widely used pesticides: azoxystrobin (fungicide), flupyradifurone, and sulfoxaflor (both insecticides)—mixed into pollen and nectar in field-realistic concentrations. We found that bees always consumed the least of sulfoxaflor-treated food, whereas consumption did not differ between other treatments or heatwave scenarios. Surprisingly, pesticide-stressed colonies performed slightly better in the future heatwave scenario in terms of reproductive fitness and survival. Sulfoxaflor consistently had the strongest negative effect, reducing survival rates, brood development, and food consumption, although effects were less severe in the future heatwave scenario.

INTRODUCTION

Pollinator decline has been heavily debated in science and beyond for the past decades.^{1,2} The alteration and fragmentation of habitats, the spread of pathogens, ongoing climate change, and the intensification of agriculture, including a massive increase in pesticide use,^{2–5} are typically discussed as main causes for the observed decline in pollinator abundances and diversity. Pollinator decline also has significant implications for human food production, as many crop species in Europe⁶ and the world⁷ depend on pollinators, especially on bees as most renowned pollinator group.^{8,9} In particular, the increased use of pesticides in agricultural landscapes but also in commercial greenhouses^{10,11} can severely affect wild and managed bees.^{6,7,12–19} Such effects include but are not limited to navigation impairment, reduced cellular health and enzyme activity, increased mortality, and changes in behavior,^{20–25} with consequences for their colonies¹⁹ and function as pollinators.^{26–28} To date, many studies have investigated the impact of pesticides on bees, including (sub-)lethal effects^{29–31} and the combined effects of different pesticides.^{32,33} Several studies have also taken into account that, for pollinators foraging in human-dominated landscapes, stress is rarely caused solely by pesticides but co-occurs with other stressors, such as infections by pathogens³⁴ or climate change. While climate change results in globally increased temperatures, it is expected to locally enhance weather extremes and, e.g., increase summer heatwaves. Heat and heatwaves can also strongly affect pollinators.^{35–44} For example, heatwaves negatively affected learning and memory in developing *Bombus terrestris*³⁸ and impaired responses to sensory stimuli.⁴⁵ Heatwaves also negatively impacted foraging in *Bombus impatiens* by reducing the proportion of successful foraging bouts, their duration, and subsequently flower visitation.³⁹ Moreover, *Bombus impatiens* colonies struggled with maintaining their nesting temperatures under heatwave conditions and, in extreme cases, were abandoned.⁴⁰ In *Bombus magnus* and *Bombus jonellus*, reproduction was also negatively affected by heatwaves.⁴⁴ Furthermore, survival and antibacterial immunity were reduced by heatwaves in *Bombus impatiens*.⁴² Similarly, mortality of *Osmia lignaria* larvae increased by 130% under severe heatwaves.⁴³ In contrast, drone production in *B. terrestris* was positively affected by mild heatwaves.⁴¹

A combined effect of stressors, e.g., extreme temperatures and pesticides, is thus likely even more harmful to bees than individual stressors, as has been hypothesized by several scientists.^{2,32} In fact, pesticide exposure can increase infections at both individual and colony levels in honeybees.^{14,34,46} Similarly, non-optimal rearing temperatures increased pesticide susceptibility in honeybee larvae,⁴⁷ and colder temperatures increased the sensitivity of overwintering honeybees to neonicotinoids.⁴⁸ Small bumblebee colonies were also more sensitive to neonicotinoids under cold stress.⁴⁹ In turn, warm wintering temperatures combined with pesticide exposure led to a 70% longevity decrease in the solitary bee *Osmia cornuta*.⁵⁰ The combined effect of pesticide and climatic stress additionally affected the movement and feeding behavior of bumblebees, but effects depended on the specific combination of temperatures and pesticide.⁵¹

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Table 1. Pesticide concentrations used

Substance	Concentration	Reference
Flupyradifurone	4300 $\mu\text{g kg}^{-1}$ (nectar)	Tong et al. ⁵³
	21.0 $\mu\text{g kg}^{-1}$ (pollen)	Tong et al. ⁵³
Sulfoxaflor	21.85 $\mu\text{g kg}^{-1}$ (average over 2 years) pollen	Jiang et al. ⁵⁴
	9.7 $\mu\text{g kg}^{-1}$ (average over 2 years) nectar	Jiang et al. ⁵⁴
Azoxystrobin	3.2 ppb in pollen	Mullin et al. ⁵⁵

Pesticide residues as found in pollen and/or nectar in the field. All data reported refer to the active substance in each pesticide. Note that we used double the concentration as cited to account for the dilution effect of mixing the pollen 1:1 with water.

To understand the combined effects of field-realistic concentrations of pesticides and heatwaves on pollinator development, feeding behavior, and longevity, we conducted a climate chamber experiment (simulating realistic daily conditions) with the buff-tailed bumble bee *B. terrestris*, a highly important pollinator species in Europe. We simulated two heatwave scenarios in climate chambers of the Model EcoSystem Analyser of the Technical University of Munich (TUMmesa, Freising, Germany)⁵²: a current heatwave scenario (10–30°C) and a future heatwave (15–35°C) (Tables S1 and S2, <https://doi.org/10.17605/OSF.IO/6BKP9>). Both scenarios were based on summer heatwaves in southern Germany. For the current heatwave scenario, we used temperature and humidity values derived from the database of the “Deutscher Wetterdienst.” For the future heatwave scenario, we used temperature and humidity values derived from the “Klima-Report Bayern 2021.” The climate chambers simulated a daily temperature, humidity, and light rhythm. Temperatures gradually increased toward the heatwaves and decreased afterward (see STAR Methods for more in-depth information).

We additionally exposed bees to field-realistic concentrations of the active substances of three widely used systemic pesticides (Table 1): flupyradifurone (used, e.g., in Sivanto Prime, insecticide against white flies and aphids; used for strawberries, tomatoes, cucumbers, etc.), sulfoxaflor (used, e.g., in Closer, insecticide against aphids and white flies; used for pumpkins, zucchini, and ornamental plants), and azoxystrobin (used, e.g., in Amistar, fungicide against rust fungi in grains, sclerotinia in rapeseed, and mildew used for peas and hops). We analyzed the combined effect of these substances in pollen and nectar and of heatwaves on food consumption, brood development, and worker longevity of *B. terrestris* microcolonies kept in the two climate chambers with the present-day and the future heatwave scenario. Our experimental time was set to cover the whole larval development, and thus longer than in most other studies with *B. terrestris*, to elucidate interactive effects of heat and pesticides across developmental stages. Moreover, we applied field-realistic pesticide concentrations based on information about residues found in pollen and nectar, whereas most previous studies used concentrations following the instruction labels of commercial pesticide solutions.

Bumble bees have difficulties decreasing their body temperature at high ambient temperatures (>35°C)^{56–58} and may therefore suffer from heatwaves. As several studies have already shown negative effects of pesticides and increased temperatures on the performance of different bee species (see earlier discussion), we hypothesized that the combined effect of pesticides and heatwaves would negatively affect brood production and worker longevity. We additionally hypothesized that these negative effects would be even more severe under the more extreme future heatwave scenario. We nevertheless expected a more substantial impact of pesticides than of heatwaves, as, unlike heatwaves, pesticides represented a direct stressor to the bees, which they could not avoid through, e.g., reducing foraging activity or foraging of different food sources in our laboratory setup. When comparing the effect of different pesticides, we hypothesized that flupyradifurone and sulfoxaflor would have the strongest effect, irrespective of the heatwave scenario, because they trigger the expression of the same nicotinic acetylcholine receptor subunits^{59,60} (as shown in *Drosophila*⁶¹) and are therefore likely similar to neonicotinoids in their effect on bees.

RESULTS

Pollen consumption

Mean pollen consumption

Mean pollen consumption did not differ between heatwave scenarios ($F_1 = 1.84$, $p = 0.177$; Figure 1). However, it did differ between treatments ($F_3 = 9.41$, $p < 0.001$). There was no significant interaction between treatments and heatwave scenarios ($F_3 = 0.38$, $p = 0.769$; Figure 1). Pollen treated with sulfoxaflor was consumed significantly less than control pollen (generalized linear (GL) hypotheses: $p = 0.008$) and flupyradifurone-treated pollen (GL hypotheses: $p = 0.061$) across heatwave scenarios (Figure 1). There were no further significant differences in mean consumption within or across heatwave scenarios (Figure 1).

Pollen consumption over time

Pollen consumption over time showed a significant interaction between heatwave scenarios and treatments ($F_3 = 6.98$, $p < 0.001$) and differed between heatwave scenarios ($F_1 = 5.18$, $p = 0.023$) and across treatments ($F_3 = 6.98$, $p < 0.001$; Table S3). Within both heatwave scenarios, pollen treated with sulfoxaflor was consumed significantly less than any other pollen (Tukey: $p < 0.001$ against all other pollen treatments). In the current heatwave scenario, pollen treated with azoxystrobin was consumed less than the control pollen (Tukey: $p = 0.007$) during the last days of the experiment (Figure S1). No further significant differences between treatments and heatwave scenarios were found (Table S3).

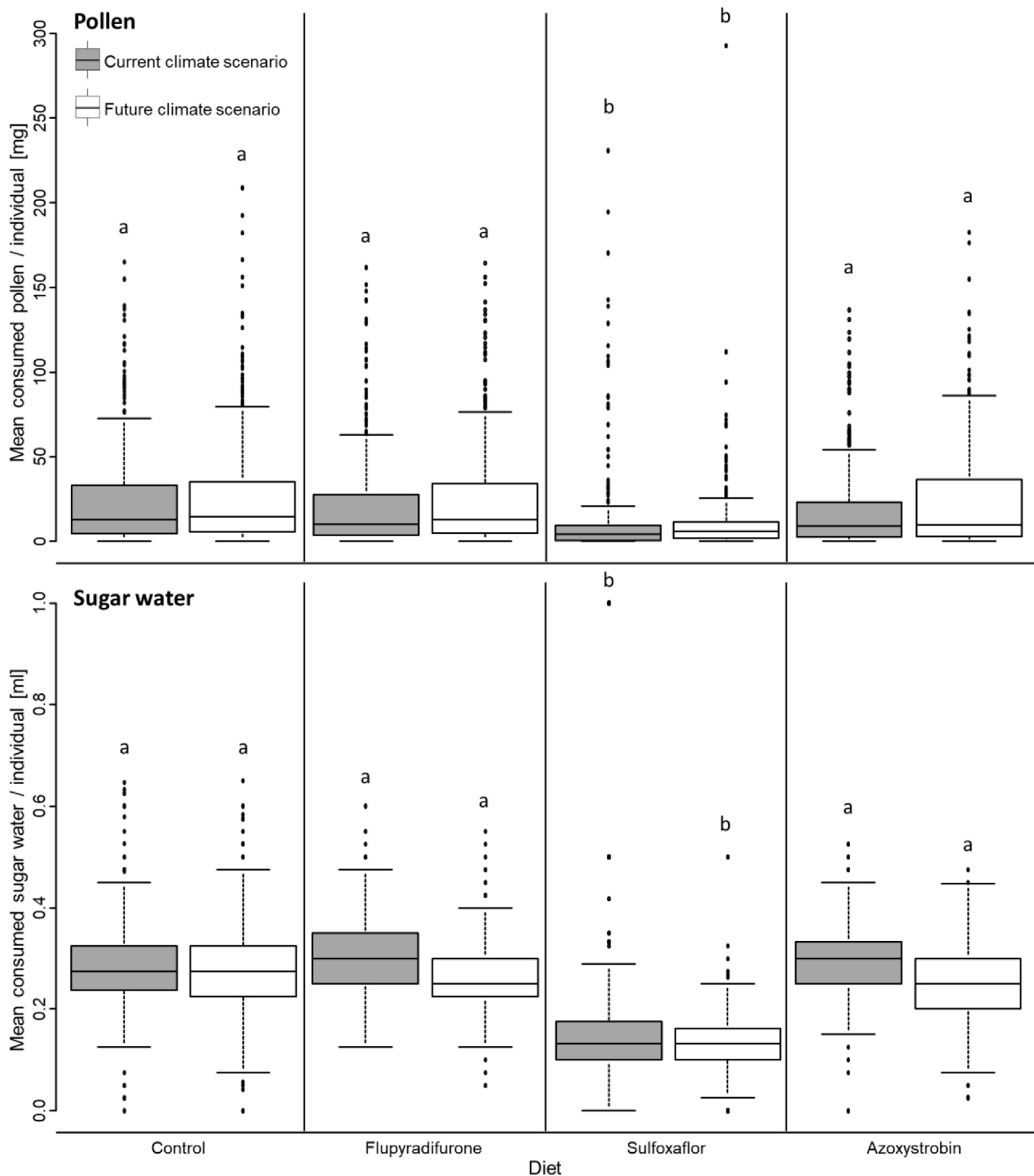


Figure 1. Mean pollen and sugar water consumption

Pollen: mean pollen consumed per individual *Bombus terrestris* worker in microcolonies fed treatments with different pesticides added and kept under different heatwave scenarios (present-day and future). Different letters indicate significant differences between groups. Sugar water: mean sugar water consumed per individual *B. terrestris* worker in microcolonies fed treatments with different pesticides added and kept under different heatwave scenarios. Different letters indicate significant differences between groups based on a post hoc test (generalized linear [GL] hypotheses).

Sugar water consumption

Mean sugar water consumption

Mean sugar water consumption differed between heatwave scenarios ($F_1 = 16.09$, $p < 0.001$) and treatments ($F_3 = 81.99$, $p < 0.001$), whereas the interaction between treatment and heatwave scenarios was not significant ($F_3 = 1.15$, $p = 0.331$) (Figure 1). The bees consumed the least of sugar water treated with sulfoxaflor (GL hypotheses: $p < 0.001$ against all other treatments), whereas consumption of control sugar water and sugar water treated with either azoxystrobin or flupyradifurone did not differ (Figure 1).

Sugar water consumption over time

Sugar water consumption over time also showed significant differences between treatments ($F_3 = 235.94$, $p < 0.001$) and heatwave scenarios ($F_1 = 20.36$, $p < 0.001$). The interaction between treatments and heatwave scenarios was also significant ($F_3 = 11.34$, $p < 0.001$). Consumption of sugar water treated with flupyradifurone and azoxystrobin was lower in the future heatwave scenario than in the current heatwave scenario (flupyradifurone $F_1 = 10.73$, $p = 0.025$; azoxystrobin $F_1 = 4.58$, $p = 0.033$; Figure S2). Consumption of sugar water treated with sulfoxaflor was slightly higher in the future heatwave scenario compared to the current scenario ($F_1 = 5.04$, $p = 0.025$; Figure S2). Overall, however, pollen treated with sulfoxaflor was consumed the least (Tukey: $p < 0.001$ against all other treatments; Figure S2). Pollen treated with flupyradifurone was consumed more over time than control pollen in both heatwave scenarios (Tukey: current $p = 0.025$; future $p = 0.006$; Figure S2). Consumption of control pollen over time did not differ between heatwave scenarios ($F_1 = 0.23$, $p = 0.63$; Figure S2). We also found no differences between the control and azoxystrobin treatments (Figure S2).

Reproductive fitness

Egg clumps, larvae, and pupae were randomly deposited by workers within the hive box. We continuously counted the number of egg clumps, larvae, and pupae without disturbing the nest, i.e., we did not move, open, or otherwise manipulate the brood cells (Figure S4).

Egg clump abundance significantly differed between treatments ($F_3 = 90.75$, $p < 0.001$). Fewer egg clumps were produced in the sulfoxaflor treatment than in the azoxystrobin (Tukey: $p = 0.003$) and flupyradifurone treatments (Tukey: $p = 0.031$) (Figure 2). Across treatments, the abundance of egg clumps ($F_1 = 0.67$, $p = 0.412$) did not differ between heatwave scenarios, whereas it was significantly affected by the interaction between heatwave scenarios and treatments ($F_3 = 49.29$; $p < 0.001$; Figure S3). Colonies treated with sulfoxaflor produced more egg clumps in the future heatwave scenario than in the current scenario (Figure S3). There was no significant effect of treatment ($F_1 = 0.96$, $p = 0.412$) or heatwave scenario ($F_1 = 0$, $p > 0.999$) and no significant interaction effect between treatments and heatwave scenarios ($F_3 = 0.19$, $p = 0.906$) on first-time appearance of egg clumps (Figure 2).

The abundance ($F_3 = 364.49$, $p < 0.001$) and first-time appearance ($F_3 = 121.23$, $p < 0.001$) of larvae also significantly differed between treatments (Figure S3; Figure 2). The least larvae were produced in the sulfoxaflor treatment (Tukey: $p < 0.001$; Figure S3), and the larvae also appeared later during both heatwave scenarios for that treatment (Tukey: $p < 0.001$) (Figure 2). Overall larvae abundance ($F_1 = 0.99$, $p = 0.320$) and first-time appearance ($F_1 = 0.82$, $p = 0.365$) did not differ between heatwave scenarios (Figure S3; Figure 2), whereas the first-time appearance was significantly affected by the interaction between heatwave scenarios and treatments ($F_3 = 5.83$, $p < 0.001$). For the sulfoxaflor treatment, first-time appearance of larvae was earlier in the future heatwave scenario than in the current heatwave scenario (Figure 2). There was no significant interaction effect between heatwave scenarios and treatments on larvae abundance ($F_3 = 0.85$, $p = 0.468$; Figure 2, Figure S3).

Pupae abundance differed between both treatments ($F_3 = 4.51$, $p = 0.004$) and heatwave scenarios ($F_1 = 22.94$, $p < 0.001$; Figure S3). Overall fewer pupae were produced in the current heatwave scenario; and, across scenarios, the fewest pupae were produced in the sulfoxaflor treatment (Tukey: $p < 0.001$, in current and future scenario) (Figure S3). There was also a significant interaction effect on pupae production ($F_3 = 6.58$, $p < 0.001$). Overall pupae abundance was higher in the future heatwave scenario but highest for the control in the current and for the azoxystrobin treatment in the future heatwave scenario (Figure S3).

Moreover, the first appearance of pupae also differed between heatwave scenarios ($F_1 = 25.31$, $p < 0.001$). Pupae appeared overall later in the current heatwave scenario than in the future heatwave scenario (Figure 2). Pupae in the sulfoxaflor treatment were always the slowest to appear compared to all other treatments (Tukey: $p < 0.001$, in the current and future scenario). However, treatment had no effect on the first-time appearance of pupae in the other three treatments ($F_3 = 0.37$, $p = 0.756$), and there was also no significant interaction between treatments and heatwaves regarding the first-time appearance ($F_3 = 0.30$, $p = 0.882$; Figure 2).

Survival

Overall survival of workers did not differ between heatwave scenarios for any of the treatments (after correction: control $p > 0.99$, flupyradifurone $p = 0.40$, sulfoxaflor $p = 0.40$, azoxystrobin $p > 0.99$; Figure 3). However, within heatwave scenarios, survival differed between different treatments (Figure 3). Under the current-day scenario, bees fed with sulfoxaflor died faster than bees fed any other treatment (after correction: versus control $p = 0.002$; vs. flupyradifurone $p < 0.001$; vs. azoxystrobin $p < 0.001$), whereas there was no clear difference between the other treatments (Figure 3). In contrast, under the future scenario, survival tended to be highest for flupyradifurone (first death after 15 days; after correction: versus control $p = 0.06$, versus azoxystrobin $p = 0.42$; before correction: versus control $p = 0.01$, versus azoxystrobin $p = 0.07$) and lowest for sulfoxaflor (versus control $p = 0.04$, versus flupyradifurone $p < 0.001$, versus azoxystrobin $p = 0.001$), whereas it did not differ between the control and azoxystrobin treatment (Figure 3).

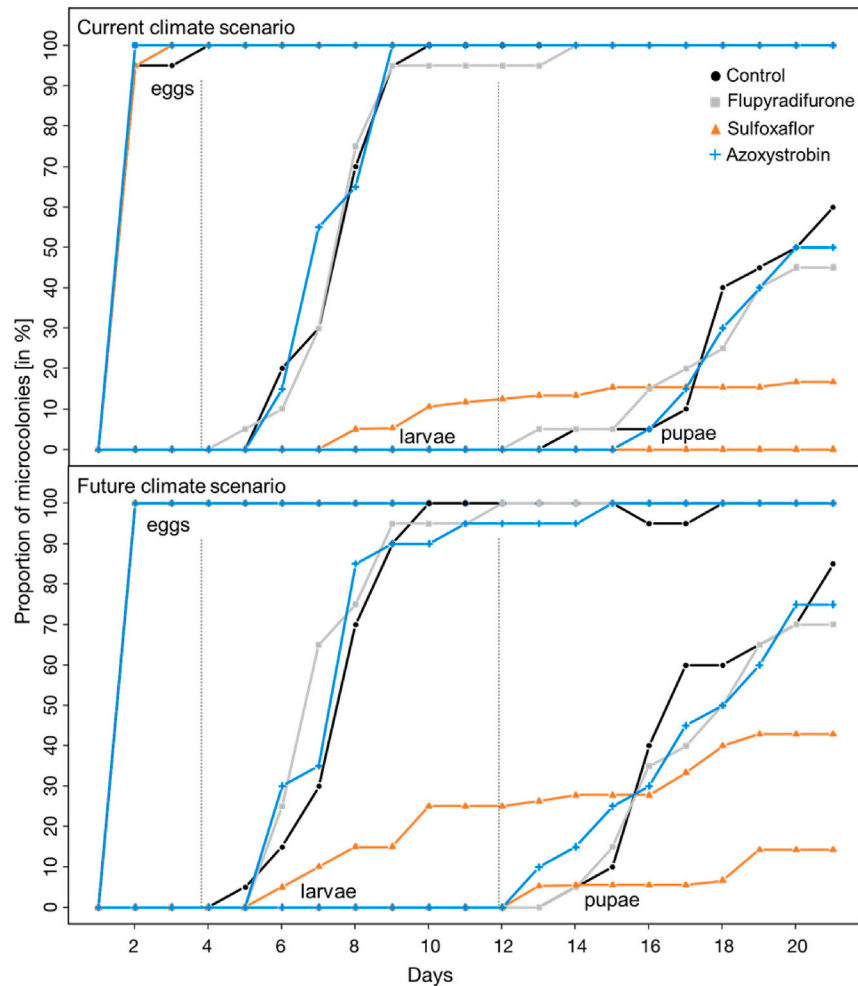


Figure 2. Reproductive fitness

Proportion of micro colonies with eggs/larvae/pupae in their nest on the corresponding day (x-axis). The proportion was calculated based on emergence times of eggs, larvae, and pupae produced by all *Bombus terrestris* microcolonies exposed to a specific treatment ($n = 160$) over the course of the experiment (21 days) fed pollen treated with different pesticides (different symbols) and exposed to two different heatwave scenarios (current [upper panel] and future [lower panel]).

DISCUSSION

Our findings demonstrate that contrary to our expectations, the combined effect of two stressors, i.e., pesticides and heatwaves, did not aggravate the effects of the individual stressors on worker longevity and brood development of *B. terrestris* microcolonies. While in particular microcolonies exposed to pollen and nectar treated with sulfoxaflor did not only decrease consumption but performed overall worse than microcolonies exposed to any other treatment, they did perform better, i.e., produced more offspring and earlier, in the future than in the current heatwave scenario. At first, this finding seems surprising as *B. terrestris* is considered a cold-temperate-adapted species that (unlike many other wild bees) can forage during cold temperatures.^{62,63} However, Sepúlveda et al.⁴¹ recently also found that *B. terrestris* drone production increased under a mild heatwave (30–32°C) scenario. The scenario used in their study is comparable to our current heatwave scenario, where colonies produced fewer drones than in the future (warmer) heatwave scenario (up to 35°C). Our findings also agree with Herbertsson et al.⁶⁴ who showed that, in southern Sweden, *B. terrestris* has increased in abundance and thus likely benefitted from a warming climate. The low sensitivity of *B. terrestris* microcolonies to heatwaves observed in our study may alternatively be explained by the use of colonies from Biobest, which have been raised under lab conditions for many generations and are predominantly sold to farmers for pollination in greenhouses.¹⁰ In fact, Velthuis and van Doorn¹¹ even suggested that these commercial colonies carry DNA from Mediterranean bees as local populations have been cross-bred, i.e., hives from Poland have been bred with hives from Italy. Therefore, *B. terrestris* may have a higher temperature tolerance than other bumble bee species, in particular under laboratory conditions, and might be generally less vulnerable to heat stress than we thought. The known negative effects of climate change on bumble bees may thus be rather due to other or more indirect effects, such as reduced or shifted resource availabilities resulting from phenological shifts,^{65–67} competition with other bee species,⁶⁴ or

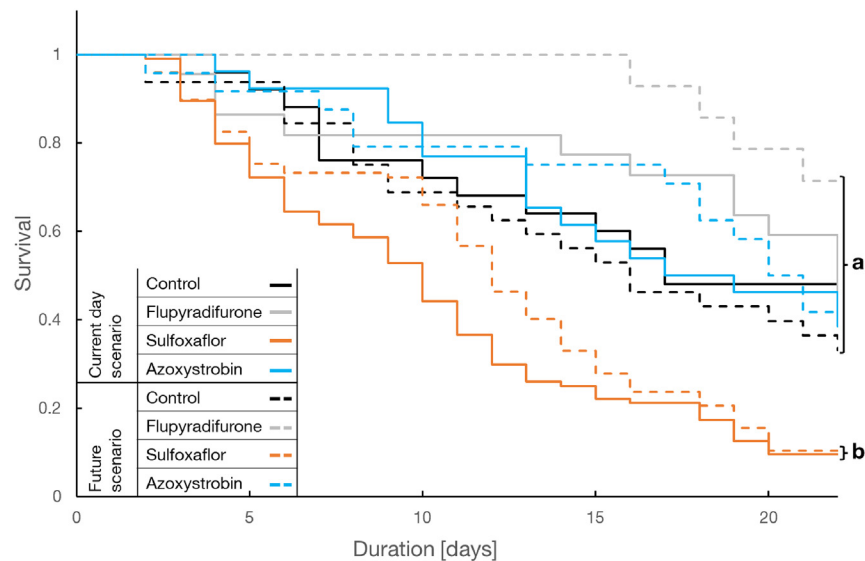


Figure 3. Survival curves

Differences in the survival of *Bombus terrestris* workers of microcolonies exposed to pollen treated with different pesticides (i.e., treatments, indicated by different colors) and kept under two different heatwave scenarios (present-day and future, indicated by solid and dashed lines, respectively). Different letters indicate significant differences between treatments and heatwave scenarios based on Kaplan-Meier survival statistics. Note that differences between treatments varied when analyzed separately for each heatwave scenario (see main text for details).

adverse effects of warmer winter temperatures on the survival of overwintering queens.^{68,69} Notably, our results might change once bees were actually forced to forage outside and fly in a heatwave, which prevents them from saving energy in response to heat stress.⁷⁰ In fact, insects show different levels of heat tolerance^{56,57,71,72} and occupy different thermal niches in which they are active.⁷³ These species-specific niches might be exceeded by heatwaves, which would lead to a reduction in flight activity and, thus, pollination. Subsequent experiments testing the effects of combined stressors should thus aim to use wild bumble bees, ideally even from different species, and test climate change effects under more natural conditions and beyond heatwaves. Note that we ran the same heatwave scenarios in the same climate chambers, instead of switching scenarios between chambers in the two experimental trials. We can, therefore, not entirely rule out an effect of the respective climate chambers. Yet, we consider it very unlikely that the specific chambers may have biased our results, as they were monitored over the entire period and had identical climatic variables (max. 1%–5% deviation from the target humidity and temperature values; Figures S5–S7).

As mentioned earlier, we observed the most significant decrease in consumption, reproductive fitness, and survival for the sulfoxaflor treatment. In some colonies fed this pesticide, brood development failed entirely, and worker behavior was heavily impacted, in particular under the current heatwave scenario. In these colonies, we observed a continuous and seemingly aimless wandering of workers and a decreased response to external vibrations (C. Nebauer, pers. observation), which typically agitate workers and cause buzzing. These results agree with other studies, which also tested the effect of sulfoxaflor (field-realistic concentrations of a commercial mixture [Transform] from Corteva: 33.702mg/20mL water) on bumble bees and honeybees and found an increase in oxidative stress and induced apoptosis in honeybees.⁷⁴ Moreover, Tamburini et al.³³ observed reduced colony size and growth with sulfoxaflor (applied as the commercial formula Closer from Corteva: 0.4L/ha), and Capela et al.⁷⁵ showed a decrease in the bees' homing ability (Closer, Corteva: 120 g/L). Siviter et al.⁷⁶ additionally found a reduction in the reproductive success of *B. terrestris* colonies kept in the field (treated with 5 µg/dm of sulfoxaflor in sucrose). However, in another study, Tamburini et al.⁷⁷ also found that honeybees were not negatively affected by field-realistic concentrations of sulfoxaflor (Closer, Corteva: 0.4 L/ha), in contrast to bumble bees.³³ Interestingly, the concentrations in the cited papers were all higher than the concentration used in our study except for Siviter et al.⁷⁶ who used lower concentrations.

Surprisingly and contrary to our hypothesis, flupyradifurone showed very different results compared to sulfoxaflor, even though both flupyradifurone and sulfoxaflor act as nAChR-antagonists and are thus comparable to neonicotinoids in their effect.⁷⁸ In fact, in previous studies, flupyradifurone negatively affected honeybees,^{31,74,79,80} whereas our *B. terrestris* microcolonies coped surprisingly well with this pesticide, particularly in the future heatwave scenario. This discrepancy may not only be explained by the different sensitivities of the various bee species studied but also by the different methods and pesticide concentrations used across studies. For example, Tosi et al.³¹ tested long-term chronic exposure with a stepwise increase of their concentrations in a flupyradifurone/sucrose solution (444–36,000 µg/kg, considered field realistic). Short-term exposure was tested by Chakrabarti et al.⁷⁴ with the commercial formulation Sivanto Prime (Bayer) solved in water (218 µL/20 mL). Single exposure was tested by Hesselbach and Scheiner⁷⁹ with flupyradifurone solved in sucrose (83 µmol/L and 830 µmol/L). Synergistic effects with fungicides were investigated by Tosi et al.⁸⁰ again with flupyradifurone and a stepwise increase of the concentration (37.5–1200 ppm, sub-lethal dosage). The concentrations used by us were mostly lower compared to concentrations used by

most studies mentioned earlier, as we selected concentrations based on pesticide residues measured in pollen and nectar and not on concentrations considered sub-lethal or applied in the field (as specified by the manufacturer).

Also contrary to our hypothesis, azoxystrobin showed little to no effect on worker longevity or reproductive fitness, respectively, although colonies consumed less of the treated pollen toward the end of the experiment in the current heatwave scenario and the sugar water in the future heatwave scenario. This finding agrees with other studies, however, which found azoxystrobin to disrupt the gut microbiota of honeybees⁸¹ but not to have a subsequent effect on colony growth or worker longevity.⁷⁷

Interestingly, colonies exposed to pesticides and, in particular, to sulfoxaflor did not only partly mitigate negative effects on reproduction but also tended to survive for longer in the future heatwave scenario than in the current one, even when compared to colonies fed the control treatment. This mitigating effect of heat stress on pesticide exposure may be explained by changes in the bees' immune activity, which might have been primed in response to heat, similar to what has been shown in honeybees in response to viral infections.⁸² In fact, Blasco-Lavilla et al.⁸³ found that a combination of heat and starvation increased the heat shock and immune gene expression and, hence, immune activity in *B. terrestris*, which increased the metabolism rate and detoxification of pesticides in the bees' bodies.

Compared to other feeding experiments,^{47,48,51,84} a 3-week period of pesticide exposure is relatively long but was necessary as we wanted to include one complete brood development cycle (egg to late-stage pupae). In fact, some positive effects of the heatwaves, e.g., higher reproductive success under the sulfoxaflor treatment, only appeared later in the cycle, and negative effects remained stable, which justifies testing over a longer time frame. It is also not unlikely that *B. terrestris* foraging in an agricultural area is exposed to pesticides over the whole colony cycle.

Limitations of the study

A limitation of our study is that we did not quantify potential pesticide or pathogen loads in the pollen used for our treatments. Actual pesticide concentrations may consequently have been even higher or other pesticides already present in our pollen may even have acted synergistically with the ones we added or been exacerbated by pathogens present in pollen or bees. Although we believe that, if at all present, pesticide or pathogen loads were low in the food-grade organic pollen used, we still cannot rule out that they might have affected our results, which renders comparisons with other studies regarding concentrations slightly unreliable. We also did not measure brood weight or size, but only the total amount of egg clumps, larvae, and pupae, which may have hidden some sublethal effects. However, we used the same pollen for all treatments, which should have led to similar effects across microcolonies. Moreover, we used queenless microcolonies, which represents a common approach for feeding experiments but has been criticized for potentially leading to erroneous conclusions.⁸⁵ Future experiments should, therefore, also test queenright colonies, ideally under more natural foraging conditions and in more different temperature scenarios to additionally take into account possible heat avoidance strategies of bumblebees.

Conclusion

Our study shows an unexpected mitigation effect of heat stress on the (negative) impact that field-realistic doses of three pesticides, in particular sulfoxaflor, have on the reproductive fitness and survival of microcolonies of the bumble bee *B. terrestris*. This might be explained by an immune priming effect of heat that accelerates detoxification mechanisms and may explain why some bee species are less sensitive to heat stress than others. Increased and more intense heatwaves represent an important aspect of climate change, but their effects on many organisms, including pollinating insects, are still largely unknown. Our results indicate that synergistic effects of different stressors are likely, but hard to predict, urging for more studies on interactions between different stressors on various organisms. We only start to understand the impacts of combined stressors and the mechanisms underlying the responses of different organisms.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Sara D. Leonhardt (sara.leonhardt@tum.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The raw data for the food consumption, brood, and survival, as well as the temperature and humidity data, will be publicly available at the date of publication. The DOI is listed in the [key resources table](#).
- All original code has been deposited at OSF and will be publicly available at the date of publication. DOI is listed in the [key resources table](#).
- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.D.L., P.P., and C.A.N.; methodology, C.A.N. and P.P.; investigation, C.A.N.; formal analysis, F.A.R. and C.A.N.; writing—original draft, C.A.N. and S.D.L.; writing—review & editing, all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
- METHOD DETAILS
 - Experimental set-up for heatwaves
 - Pesticides and treatment preparation
 - Experimental procedure
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2024.111050>.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Raw data: food consumption, brood, survival	This paper	https://doi.org/10.17605/OSF.IO/6BKP9
Raw data: temperatures, humidity	This paper	https://doi.org/10.17605/OSF.IO/6BKP9
Code – for statistical analysis (R version 4.1.2)	This paper	https://doi.org/10.17605/OSF.IO/6BKP9
Other		
Deutscher Wetterdienst and Klima-Report Bayern 2021	https://cdc.dwd.de/portal/	Online databank

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

In total, 18 mother colonies of *Bombus terrestris* (Biobest, Westerlo, Belgium) were purchased and kept in a climate chamber at 25°C, 60% RH, and a 12/12 h light/dark cycle prior to the experiment. They were provided with polyfloral honeybee-collected pollen (Aspermühle, Goch-Asperden, Germany) and API-Invert, a mixture of sucrose, fructose, and glucose (Südzucker AG, Mannheim, Germany) *ad libitum*.

METHOD DETAILS

Experimental set-up for heatwaves

The experiment was conducted twice in 2022. The first round took place in June–July, and the second in August–September. The experiment was conducted inside two climate chambers at the TUMmesa ecotron facility (TUM Model EcoSystem Analyser, Freising, Germany),⁵² each capable of simulating different climatic scenarios. The experiment simulated climate conditions, including a summer heatwave, of southern Germany. One chamber simulated conditions and a heatwave as experienced nowadays (current climate; 10–30°C), the other chamber simulated conditions and a heatwave as expected in 50 years (future climate; 15–35°C) (Tables S1 and S2). Both chambers were programmed to run at a predetermined temperature and humidity program over a period of three weeks, including one heatwave of 10 days (4 days at max. temperatures). The values used for the climate scenarios were calculated based on hourly temperature and humidity data retrieved from the ‘Deutscher Wetterdienst’⁸⁶ for the reference period 1971–2000 for the months June–July and adjusted to fit expectations described in the ‘Klima-Report Bayern 2021’⁸⁷ for current (i.e., 2021–2050; +1°C) and future (i.e., 2071–2100; +3,8°C) conditions. Exceptionally warm days (i.e., days with temperature levels above the 98th percentile within 31 days, as opposed to regular days) were identified, and the corresponding temperature and humidity values were used to design the heatwave simulation. Temperature and humidity oscillated in a regular day and night cycle, gradually increasing towards the heatwave and decreasing afterward. We also included one day before and after the heatwave, which heated up and cooled down more rapidly than the slow oscillations of the other days. Sunrise and sunset were simulated with a gradual increase/decrease of light during the appropriate times. The artificial climate and light cycle were modelled after an average June and July in Bavaria, southern Germany. These climate/heatwave scenarios were based on the data cited above and designed by Paula Prucker (Prucker et al. *unpublished*). Climate chambers always simulated the same climatic conditions and heatwaves across both experimental trials.

Pesticides and treatment preparation

Azoxystrobin (CAS 131860-33-8) and flupyradifurone (CAS 951659-40-8) were purchased from Sigma Aldrich (Taufkirchen, Germany), and sulfoxaflor (CAS 946578-00-3) from VWR (Ismaning, Germany). The concentrations used in this experiment were determined based on concentrations reported by previous studies for pesticide residues in pollen and nectar (Table 1). Azoxystrobin (as, e.g., used in Amistar®) is commonly applied to control different rust fungi in grains, sclerotinia in rapeseed, and mildew in peas and hops. Flupyradifurone (as e.g., used in Sivanto Prime®) is an insecticide targeting white flies and aphids in various crops, including strawberries, tomatoes, and cucumbers (and more). Sulfoxaflor (as, e.g., used in Closer®) is applied against aphids and white flies in pumpkins, zucchini, and ornamental plants.

The different treatments (pollen and nectar) were prepared three days before the experiment. A total of 8 kg of pollen was obtained from Aspermühle (Goch-Asperden, Germany) and ground into a fine powder (Graef Kaffemühle CM 800, Arnsberg, Germany). It was then divided into four treatments (2 kg each; HDPE, 2 L, VWR, Ismaning, Germany). The active substances (Table 1) were mixed with the pollen powder and stored in a closed container during the experiment. API-Invert was mixed with the respective tested substance to obtain the nectar treatment and stored in glass bottles (2 L, Duran, VWR, Ismaning, Germany). Pure unmodified pollen and API-Invert were used for the control group. The treated and control pollen was mixed with water in a 1:1 ratio to create a homogenous paste. Dilution through adding water to our pollen paste was taken into account by adding double the concentrations of substances as cited in Table 1.

Experimental procedure

The mother colonies were partitioned into queenless microcolonies, which were housed in two-chambered wooden nest boxes comprised of a nesting and a foraging chamber (125 × 145 × 105 mm³ per chamber; Figure S4). The outside walls of the box contained holes covered with wire mesh to facilitate air circulation. The bottom of the boxes was filled with a 2-cm layer of cat litter (Bio-Catolet, Albrbruck, Germany). The boxes were covered with transparent Plexiglas lids with holes for easy access to the colony. Each microcolony comprised 20 worker bumble bees from a single mother colony (workers from different colonies were not mixed). In total, 160 microcolonies were used (80 during June–July and another 80 during August–September). Of the 80 colonies per experiment, 40 were placed into TUMmesa chamber 2 (current climate/heatwave) and the other 40 into chamber 3 (future climate/heatwave). The microcolonies were randomly assigned to treatment groups. Each climate chamber contained ten microcolonies per treatment plus the control.

After the bees were transferred into the TUMmesa chambers, they had an acclimatization phase of 72 h, where they were left undisturbed with *ad lib.* access to untreated pollen and API-Invert. Within this timeframe, the bees established a dominant worker which started laying eggs and produced male brood. Once the 72 h had elapsed, all dead bees were replaced with living ones, and the feeding experiment started and ran for 21 days. This time frame was chosen to assess the three stages of brood development (egg, larvae, pupae). As our experience has shown that microcolonies vary in development speed, we considered three weeks enough time for all the colonies to develop pupae.

The treated or control pollen paste was presented to the colonies in a petri dish, which was placed in the foraging chamber of their boxes. It was renewed daily, and the quantity was adjusted to colony growth to ensure unlimited access (approx. 0.5 g was provided daily at the start, which then increased up to approx. 5 g throughout the experiment). The Petri dishes were weighed daily to measure pollen consumption. To account for weight loss due to evaporation, we subtracted the weight of another dish with the same treatment in a control box without bumble bees. The nectar/pesticide mix was provided in a Falcon tube (15 ml with two holes at the 2 ml mark) in the foraging chamber. The tubes were refilled, and the consumed API-Invert was recorded daily. We calculated the mean individual consumption per bee by dividing the daily consumption of pollen and nectar of each microcolony by the number of worker bumble bees found in the respective microcolony on that day to take any early deaths of workers into account.

Additionally, brood development was monitored daily by counting the number of egg clumps, larvae, and pupae without disturbing the brood. This was done by observing the nest from above and gently removing any wax the bees might have used to cover the brood. All bees that died during the experiment were also recorded (and no longer replaced after the acclimatization phase). After the experiment had concluded, all nests were additionally dissected to count the accurate brood amounts, which was not possible during the experiment.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data was analysed using R version 4.1.2.⁸⁸ First, the normal distribution of all parametric response variables was tested with a Shapiro-Wilk test and homogeneity of variances with a Levene's test (car package⁸⁹). We then tested for differences in the mean individual consumption per bee of pollen and nectar between the four treatments and two heatwave scenarios using generalized mixed effect models (GLMM, lme4 package⁹⁰) with 'mother colony' included as a random factor. We also tested for a potential interactive effect between heatwaves and treatment. Additionally, we used generalized additive mixed effects models (GAMM, gamm4 package⁹¹) to test for differences in pollen and nectar consumption (per individual and day) over time, with 'microcolonies' nested in 'mother colony' included as random factors. The GAMM model was chosen to take into account the non-linear variation in daily pollen consumption over time. This was necessary because pollen consumption changed with the number of larvae present. All colonies started without brood and only started laying eggs after the dominant worker had been established. Data was square-root transformed to achieve normal distribution.

Kaplan-Meier survival statistics were used to assess differences in the survival rates of bumble bee workers in microcolonies. All treatments were tested against each other and between heatwave scenarios using the median survival times (R-packages: *KMsurvpackage*⁹²; *survival*⁹³). The α -levels of *p*-values were adjusted using Bonferroni correction.

Finally, the effect of treatments and heatwaves, as well as their interaction on reproductive fitness, i.e., the daily number of egg clumps, larvae, and pupae produced over the course of the experiment, was compared using GAMMs. We additionally investigated differences in the emergence time (i.e., first-time appearance of eggs, larvae, or pupae) also with GAMMs. 'Microcolony' nested in 'mother colony' were again included as random factors. The first-time appearance was entered as a binomial variable, i.e., whether eggs/larvae/pupae were present (1) or not (0). The significance threshold was at 5%.