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Sub-lethal but potentially devastating - The novel insecticide flupyradifurone impairs collective brood care in bumblebees

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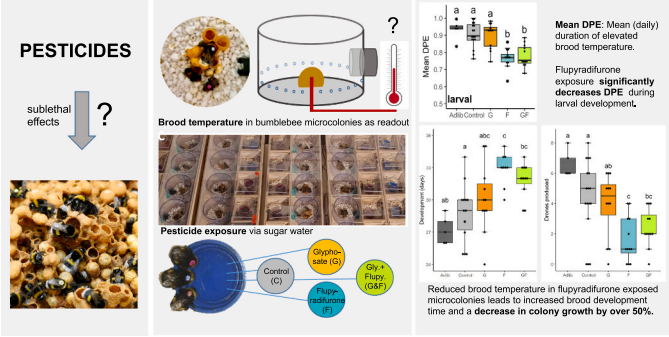
HIGHLIGHTS

- Risk assessments fail to detect sublethal effects of pesticides on pollinators.
- Novel bioassay tests sublethal effects of stressors in bumblebee microcolonies.
- Flupyradifurone impairs collective thermoregulation in bumblebee microcolonies.
- Disruption of brood care reduces reproductive output by 50%.

GRAPHICAL ABSTRACT



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ABSTRACT

The worldwide decline in pollinating insects is alarming. One of the main anthropogenic drivers is the massive use of pesticides in agriculture. Risk assessment procedures test pesticides for mortality rates of well-fed, parasite free individuals of a few non-target species. Sublethal and synergistic effects of co-occurring stressors are usually not addressed. Here, we present a simple, wildly applicable bio-essay to assess such effects. Using brood thermoregulation in bumblebee microcolonies as readout, we investigate how this collective ability is affected by long-term feeding exposure to the herbicide glyphosate (5 mg/l), the insecticide flupyradifurone (0.4 mg/l) and the combination of both, when co-occurring with the natural stressor of resource limitation. Documenting brood temperature and development in 53 microcolonies we find no significant effect of glyphosate, while flupyradifurone significantly impaired the collective ability to maintain the necessary brood temperatures, resulting in prolonged developmental times and a decrease in colony growth by over 50 %. This reduction in colony growth has the potential to significantly curtail the reproductive chances of colonies in the field. Our findings highlight the potentially devastating consequences of flupyradifurone use in agriculture even at sub-lethal doses and underline the urgent need for improved risk assessment procedures.

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1. Introduction

Insect pollinators are key not only to the preservation of biodiversity but also to the worldwide food supply (Gallai et al., 2009; Klein et al., 2007). The ongoing 'pollinator crisis', the well-documented worldwide decline in abundance and diversity of pollinators (Biesmeijer et al., 2006; Hallmann et al., 2017; Hochkirch, 2016; Powney et al., 2019; Seibold et al., 2019), is highly alarming. Its main drivers are of anthropogenic background (Potts et al., 2010; Vanbergen and IP initiative, 2013) and include climate change and environmental pollution (Kerr et al., 2015; Marshall et al., 2018; Potts et al., 2015), urbanization and intensified agriculture (Goulson et al., 2015; Hendrickx et al., 2007; Kennedy et al., 2013; Winfree et al., 2009), and the use of pesticides and other agrochemicals (Godfray et al., 2015; Goulson, 2013; Rortais et al., 2005). It is the combination of these anthropogenic stressors with natural stressors like diseases, parasites, competition and resource limitation is believed to be responsible for the rapid decline of wild pollinators worldwide (Goulson et al., 2015). For pollinators in agricultural landscapes, the combined occurrence of resource limitation due to lack of wild flowers and pesticide exposure is especially relevant (Castle et al., 2023; Requier et al., 2020; Roulston and Goodell, 2011; Samuelson et al., 2018; Scheper et al., 2014). Resource limitation and ensuing nutritional deprivation has been shown to increase the susceptibility to adverse effects of pesticides (Goulson et al., 2015; Stuligross et al., 2023; Stuligross and Williams, 2020; Weidenmüller et al., 2022). In order to provide a realistic assessment of the costs associated with pesticide use in agriculture for pollinating insects, risk assessment procedures need to test both lethal and sublethal effects of agrochemicals under conditions of resource limitation (Goulson et al., 2015). However, to date, no such standardized risk assessment procedures exist.

Pesticide use has increased steadily ever since the industrialization of agriculture (Aktar et al., 2009), reaching close to 4.2 billion kilograms applied globally in 2019 (FAOSTAT, 2021), comprising >1600 different chemical substances (Horrigan et al., 2002). Regularly, new pesticides are introduced to the market and the regulatory procedures behind this rely on Environmental Risk Assessments (ERA). ERAs focus on acute toxicity screening of the active substance only (LD_{50}, 24 or 48 h after exposure) in a small subset of non-target species acting as surrogate test species (Sánchez-Bayo and Goka, 2014). Passing the ERAs results in a ten-year license for the new substance (Topping et al., 2020). Usually, a single substance is tested on well-fed, healthy individuals. Combinatorial effects of different substances and the influence of other stressors are disregarded and sublethal and long-term exposure effects are not assessed.

The most commonly used species in toxicological studies on pesticide effects on bees is the honeybee, *Apis mellifera* (Franklin and Raine, 2019). However, as social bee with a perennial colony life cycle, very large colony sizes and mass foraging, *A. mellifera* may differ considerably in pesticide exposure and susceptibility from wild bees and is not necessarily suitable as a surrogate species (Thompson and Hunt, 1999). Wild bumblebees are among our most important pollinators and have been shown to be more sensitive to some pesticides than honeybees (Mundy-Heisz et al., 2022; Rundlöf et al., 2015). They increasingly serve as surrogate for wild bees in ecotoxicological studies (Gradish et al., 2019). Most bumblebee species have an annual colony cycle, and the fast build-up of a strong worker force during a short growth season is essential for the reproductive success of a colony (Owen et al., 1980).

Bumblebees have the unusual ability to expedite the development of their brood *via* active thermogenesis, maintaining brood temperature at 30–35 °C (Grad and Gradišek, 2018; Heinrich, 1979; Weidenmüller et al., 2002), where brood survival is high and development fast (Weidenmüller et al., 2022). Collective incubation is energy costly, requiring sufficient amounts of nectar (Silvola, 1984; Vogt, 1986). Resource limitation is therefore a highly relevant and common stressor for wild colonies (Carvell et al., 2017), especially in agricultural landscapes (Requier et al., 2020; Samuelson et al., 2018). A limitation in nectar supply weakens a colony's collective incubation ability, resulting in suboptimal brood temperatures, increased developmental times and consequently reduced colony growth and a loss of reproductive chances (Grad and Gradišek, 2018; Heinrich, 1996; Owen et al., 1980; Schmid-Hempel and Schmid-Hempel, 1998; Weidenmüller et al., 2022). Any impairment of collective thermoregulation will have serious fitness consequences for a colony, and stability in brood temperature can serve as a proxy for colony health, growth and fitness. Recent studies have used the collective thermoregulation ability of queenright bumblebee colonies as a readout and have documented sublethal effects of neonicotinoids (Crall et al., 2018) and of Glyphosate (Weidenmüller et al., 2022). Importantly, the impairing effects of Glyphosate on collective thermoregulation were evident only under resource limitation (Weidenmüller et al., 2022), again underlining the importance of testing colonies and individuals under such conditions. Developing new risk assessment strategies that include exposure to natural stressors while testing for sublethal effects of pesticides and linking individual level effects to possible colony or population impairment is challenging but crucial (Siviter et al., 2023). Testing large queenright colonies is extremely time and cost consuming, as high inter-colony variability (Bloch, 1999) often impedes standardization and necessitates large sample sizes. Bumblebee microcolonies consisting of only a few workers and brood offer an alternative.

The aim of this study is twofold: First, we present a simple, well controlled bioassay that can easily be used to test the risks of xenobiotic substances on bumblebee microcolonies under resource limitation. Second, we use this assay to measure the effects of the novel insecticide Flupyradifurone and the herbicide Glyphosate on microcolonies, building on the results for exposure effects on whole colonies by Weidenmüller et al. (2022) for Glyphosate and Crall et al. (2018) for neonicotinoids.

Glyphosate (GLY) is the most commonly used herbicide worldwide (Benbrook, 2016; Duke and Powles, 2008). Targeting the shikimate pathway (Duke and Powles, 2008), a biosynthesis pathway found only in plants and microorganisms (Herrmann and Weaver, 1999), it was long believed to be harmless for terrestrial arthropods (EFSA, 2015). However, evidence for sublethal effects of GLY on the behaviour of *A. mellifera* (reviewed in Farina et al., 2019) and wild bees (Belsky and Joshi, 2020; Franklin and Raine, 2019; Seide et al., 2018; Weidenmüller et al., 2022) has been accumulating, possibly mediated by impairments of the gut microbiome, the immune system and of general health (Blot et al., 2019; Motta et al., 2018; Motta and Moran, 2020).

Flupyradifurone (FPF), a butanolide insecticide, was introduced to the European and US market in 2015 and is now available worldwide (Nauen et al., 2015). Similar to neonicotinoids, FPF binds to the nicotinic acetylcholine receptors (NAChRs) in the insect nervous system (Nauen et al., 2015). While FPF has passed the ERA and has been described as relatively 'bee safe' (Carleton, 2014), recent studies document sublethal effects of FPF-exposure on *A. mellifera* (behaviour: Hesselbach and Scheiner (2018); Tan et al. (2017); Hesselbach et al. (2020); Tosi et al. (2021); cellular stress and immune gene expression: Al Naggar and Paxton (2021); Chakrabarti et al. (2020); larval mortality: Al Naggar and Baer (2019)), and on bumblebees (gut microbiome: Zhang et al. (2022); learning, memory and feeding motivation: Siviter and Muth (2022)).

We investigate the effects of these two pesticides on collective brood thermoregulation in bumblebee microcolonies.

2. Materials and methods

2.1. Colonies

Thirteen commercially reared *Bombus terrestris* colonies were purchased from Koppert Biological Systems. Upon arrival, colonies were transferred from their shipping boxes into wooden nest boxes under red light. Nest boxes consisted of a nest chamber and a feeding chamber

each (both 26 × 20 × 10 cm) connected *via* two holes in the adjacent walls. The nest box floor was covered with unscented non-clumping cat litter. Each chamber was covered by a translucent Perspex lid with a central cutout (5.5 × 17 cm) covered by a second lid, allowing easy access to the colony. Colonies were provided *ad libitum* access to sugar water (sucrose 50 % w/w, prepared fresh every week) in a Petri dish in the feeding chamber. Pollen was provided daily; about one tablespoon of dried, honeybee-collected pollen (Holtermann KG, Brockel, Germany) fed directly into the nest. Newly emerged workers, easily identifiable due to their greyish coloration, were marked daily with numbered plastic tags (Opalithplättchen; Holtermann KG, Brockel, Germany). Colonies were maintained in a climate-controlled room at 22 °C and 40–50 % relative humidity and a 16/8 light/dark regime.

2.2. Microcolonies

Microcolonies are groups of queenless workers (Fig. 1A); workers in this situation start laying unfertilized eggs that develop into drones. A microcolony (MC) setup consisted of a circular nest chamber (Perspex wall; diameter 8 cm; height 6 cm) connected to a wooden foraging chamber (10 × 8 × 6 cm) *via* a 4 cm tube (diameter 1 cm; Fig. 1B). A small hole drilled centrally into the wooden floor of the nest chamber served to thread the tip of a thermocouple into the nest chamber such that it protruded 1 cm into a central wax mound (1 g wax; shaped into an elongated hemisphere mimicking bumblebee pupae, made from canopy wax collected and merged from all donor colonies and frozen before use). Setup floors were covered with cat litter; the foraging chamber was equipped with a ventilation hole (2 cm) covered with a metal mesh; the nest chamber had 23 small ventilation holes 1 cm above the ground. Five microcolony setups were placed adjacent to each other in a wooden tray (52 × 21 cm, with ventilation holes around the outer edge) and covered by a Perspex lid. Circular cutouts (4 cm diameter) covered by small Perspex lids (7 × 7 cm) above each nest and foraging chamber allowed easy access (Fig. 1C).

A MC was initiated by selecting five young, marked workers (aged 1–4 days) from five different donor colonies. Using workers from five different donor colonies controlled for genetic differences between the donor colonies by equalizing the genetic background between the MCs. Each group of five workers was placed in one MC setup. 58 MCs were established. MCs were checked daily to record the status of the brood.

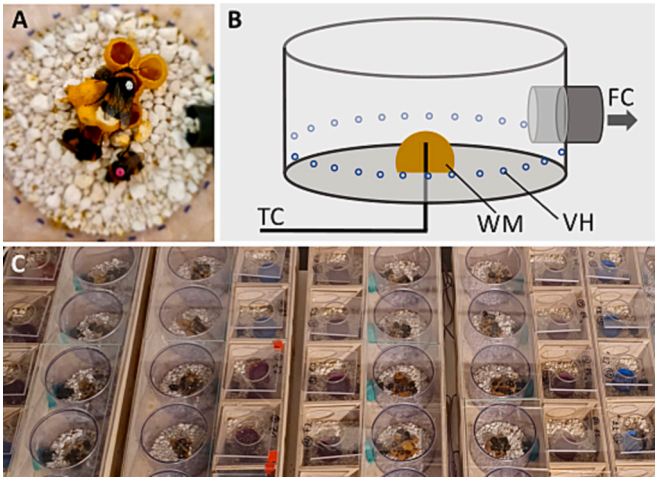

Fig. 1. Microcolony setup. A: Groups of five young, individually marked *B. terrrestris* workers from five different donor colonies were maintained in circular nest chambers. B: Perspex nest chamber with ventilation holes (VH). A thermocouple (TC) protruding through a hole in the floor into a central wax mound (WM) allows to measure brood temperatures. Nest chambers connected to a foraging chamber (FC; not shown). C: Multiple microcolonies (circular nest chambers connecting to square foraging chambers).

Every MC proceeded to lay eggs on the provided wax mound (within 6 to 10 days after MC initiation). Eggs were clearly detectable, since egg cells protruded from the smooth surface of the wax mound and since the fresh wax covering the eggs differed in colour from the darker canopy wax used to create the wax mound. For each MC the date of first oviposition was termed *day 0*. All measurements of developmental time and total length of the experiment are in reference to this day 0. MCs were maintained in the same room as the donor colonies, at 22 °C and 40–50 % RH. MCs were checked daily for the occurrence of eggs, pupae, number of emerged drones, and dead workers. Drones were removed and frozen at −20 °C within 24 h of their eclosion. Dead workers were removed within 24 h, frozen, and immediately replaced with a young, marked worker (1–4 days old).

2.3. Feeding regime

All 58 MCs had *ad libitum* access to pollen: fresh pollen bread (ground-up honeybee-collected pollen mixed with sugar water and frozen in portions of ~0.5 g – enough for there to always be left over pollen the next day) was provided daily in the nest chamber; old, dried-up pollen bread was removed. Fresh sugar water was provided daily in a dish in the foraging chamber.

Five MCs received sugar water *ad libitum* throughout the experiment, *i.e.* their feeding dishes always contained sugar water (sucrose 50 % *w/w*). While our main focus was on the effect of pesticides on brood development in microcolonies under conditions of resource limitation, data gathered in these five *ad libitum* MCs served as a baseline for our analysis.

The remaining 53 MCs were maintained under sugar water limitation, receiving 2 ml sugar water (with or without an added pesticide depending on treatment, see below) once a day at 2 pm, four days a week (Monday to Thursday), and 6 ml of the same sugar water at 2 pm on Fridays. This volume was chosen based on the results of a trial run with three MCs of five workers and brood each in which we tracked the brood temperature after providing them with different quantities of sugar water for periods of 24 h. During this trial, it became evident that while 3 ml of sugar water provided five workers with enough energy to keep the brood at elevated temperatures for 24 h, providing 2 ml of sugar water resulted in brood temperatures falling and dropping to room temperature after roughly two-thirds of the time (~16 h), demonstrating that this amount of sugar water does not provide sufficient energy for five workers to maintain their brood at constant high brood temperature for 24 h (for details see Fig. S1). The sugar water volume of 2 ml was therefore used to simulate intermittent resource limitation without causing starvation. As a result of our feeding regime, the 53 resource limited MCs experienced a few hours of resource limitation on five out of seven days, with the longest 'gap' in resource availability experienced once a week, on Sundays.

2.4. Treatments

This experiment was designed as a blind study: all sugar water solutions were prepared fresh weekly by a student helper, labelled C—F, and refrigerated; experimenters were unaware which feeding solution contained which treatment until data had been analysed.

The 53 sugar water limited MCs were randomly assigned to one of four sugar water treatment groups: Control (C; $n = 17$); Glyphosate GLY (G; $n = 12$); Flupyradifurone FPF (F; n = 12) and Combinatorial (GF) containing both GLY and FPF (n = 12). Data were collected in two replicas of this experiment between October 2020 and March 2021. To ensure genetic similarity between all MCs, all donor colonies stemmed from the same commercial provider and all MCs consisted of five bees from different donor colonies; to further secure high genetic similarity between all other MCs and Control MCs and across replicas, the first replica contained nine Control MCs, the second replica another eight (Table S1).

MCs in the Control group received untreated sugar water (sucrose 50 % w/w). MCs in the GLY treatment group (G) received sugar water containing 5 mg/l GLY. For a stock solution with a GLY concentration of 5 mg/ml, GLY (Sigma Aldrich, USA Lot: BCBW9283) was dissolved in distilled water. Until further use, stock solutions were kept frozen at −20 °C. 1 ml defrosted stock solution was filled up with sugar water (sucrose 50 % w/w) to a volume of 1 l. MCs in the FPF treatment (F) received sugar water with a FPF concentration of 0.4 mg/l. For a stock-solution pure FPF (powder, Sigma Aldrich, USA Lot: BCCB1463) was dissolved in distilled water to obtain a concentration of 0.08 mg/ml. Stock solutions were kept at −20 °C. For the weekly preparation of the treatment, 1 ml of stock solution and an additional 0.2 ml of distilled water (to match the combinatorial treatment) were filled up with sugar water (sucrose 50 % w/w) to a volume of 200 ml, resulting in the desired concentration of 0.4 mg/l. MCs in the combinatorial treatment (GF) were fed with sugar water containing both GLY and FPF in the same concentrations as the single substance treatment groups (5 mg/l GLY and 0.4 mg/l FPF). This treatment solution was prepared by using 1 ml of FPF stock-solution and 0.2 ml of GLY stock-solution and filling it up with sugar water to a volume of 200 ml.

The concentrations for the two pesticides were chosen in line with recent studies in this field which investigate sub-lethal effects on *Apis spp.* and *Bombus spp.* (Table S2), as well as with documented field-realistic residues of glyphosate found in organic honey (4,6 mg and 7,1 mg per kg; (Institut für Hygiene und Umwelt - Hamburg, 2019)).

2.5. Recording brood temperature

Brood temperature was recorded with a thermocouple (Type E, OMEGA, Germany) inserted into the wax mound provided centrally in each MC (Fig. 1B). Temperature data were logged once per minute, from the first day after assembly of MCs to termination. For the first round of experiments (Rep.1; 18 MCs), thermocouples were connected to ARDUINO UNO 3 boards equipped with a 4-channel thermocouple interface (CN0391-ARDZ shield, Analog Devices) running with a custom script, allowing to log the temperature of four thermocouples into a csv-file once per minute. In the second round of experiments (Rep.2; 40 MCs) the same thermocouples were used, now connected to two 16 Channel Thermocouple Temperature Data Loggers (OM-CP-X16TCTEMP-A2, OMEGA) allowing data logging for 16 MCs simultaneously per device. Additionally, eight MCs were connected to ARDUINO UNO as before. Temperature was again logged at a frequency of one reading per minute.

2.6. Microcolony termination and dissection

Each MC was terminated and dissected on day 35 after eggs were first detected in this MC (day 0). Workers were frozen at −20 °C. Body sizes of the frozen workers were collected by measuring the widest distance of the thorax with a calliper and then using the mean of three measurements. The brood was carefully dissected, the number of eggs was quantified, and all larvae and pupae were sorted on grid paper for size categorisation. In this process, sometimes larvae/pupae were discovered that had turned black and were obviously no longer alive. In all cases these individuals were in the process of pupating or had just pupated. They were removed and their number noted (they were found in seven of the F-MCs and two GF-MCs). Live brood was categorised, counted, photographed and weighed. Larvae and pupae were categorised according to development status or size. Larvae grow in size and weight with every instar (Cnaani et al., 2002). For comparison between treatments, larval categories were set to 'small' ($< 3 \times 3$ mm), 'middle' ($>3 \times 3$ mm & $< 9 \times 9$ mm) and 'large' ($>9 \times 9$ mm). Large larvae found within a pupal cocoon rather than a wax cell were counted into the first pupal stage. Six categories for pupal development were determined: first stage: larval shape; second stage: adult shape, completely white; third stage: adult shape, body white with red or brownish eyes; fourth stage: body white with black eyes; fifth stage: body dark, wingless; sixth stage: wings, ready to eclose. These six stages are a simplified approach to the twenty pupal stages proposed by Tian and Hines, 2018. The measured live brood weight does not include the drones that had eclosed before the termination of the experiment (and counted under 'drones emerged') but rather shows how much brood was still in the "pipeline" per MC.

2.7. Data analysis

To analyse brood temperature and its impact on brood developmental time, we used a temperature threshold of 25 °C. Brood temperatures above 25 °C were assessed as indicating active thermogenesis and incubation by the workers, resulting in brood temperatures elevated well above room temperature (21 °C - 23 °C). Bumblebee colonies maintain their brood at 30–35 °C (Grad and Gradišek, 2018; Heinrich, 1979; Weidenmüller et al., 2022), however, the readout of absolute brood temperature as recorded with the thermocouples in the wax mound was not used for analysis, as workers laid their brood on different positions on the wax mound (side/tip) and as, with increasing age and amount of brood, the brood itself 'grew' away from the sensor, resulting in temperature readouts differing between MCs and decreasing over time. Using the 25 °C threshold as a reliable indicator of incubation activity, we analyse the daily proportion of time that MCs maintained their brood at elevated, above threshold temperatures, further termed *DPE* (daily proportion of elevated temperature).

Cumulative DPE values served as a measure for the total thermal energy invested into brood from egg to first pupation and first emergence of an adult drone. The highest possible daily DPE value is 1 (corresponding to a full 24 h of uninterrupted incubation activity by the workers resulting in brood temperature constantly above the 25 °C threshold). Therefore, the highest possible cumulative DPE across the whole experimental time per MC is 34 (day 35 was excluded, because MCs were terminated mid-day on day 35).

To analyse brood development time in MCs, the time (in days) between first oviposition and first emergence of a drone was analysed. From each MC, we thus used developmental time data for the first emerged drone only, as for all subsequently emerging drones, oviposition and developmental time could not be assessed with the same amount or reliability. For MCs that did not produce a single drone during the 35 days of observation, no data on the total development duration is available. They were therefore excluded from the analysis of total brood developmental time. Analyses of the dissected brood account for any drones that would have eclosed in the following days.

In order to investigate the possible effects of the pesticide treatments during different phases of brood development, total brood development time was split into a larval (pre-pupal) and a pupal phase. Again, these categories only relate to the developmental progress of the first eggs that were apparent in a MC. This was done individually for each of the MCs. The larval phase was determined to range between the first day of detected eggs until the first day a pupation was detected in that MC, and the pupal phase followed seamlessly from first pupation to first day an adult drone emerged in this MC. The larval phase thus includes the egg phase because it was very hard to visually determine when the first larvae had actually eclosed.

To assess reproductive output of MCs, we analysed the number of drones that had emerged within the 35 days following first oviposition and the number of brood (larvae & pupae) left in each of the brood categories at time of termination of a MC on day 35 following first oviposition.

2.8. Statistical analysis

All 58 MCs of five groups (*ad libitum* and the four resource limited treatment groups: Control, F, G, FG) were included in the statistical analyses.

Using X^2-tests, we analysed whether there was a difference between groups in how many workers died and were replaced throughout the experiment and whether the mean worker size of the five workers in a MC differed between groups.

For the effect of resource limitation and pesticide treatment on DPE over the full brood development duration (first oviposition to first drone eclosion or last full day (34) of the experiment), the same dataset was used in two different ways.

First, DPE was turned into a binomial readout, scoring 1 for a full day over 25 °C (DPE-value of 1) and 0 for any day that was not consistently above 25 °C (DPE-value <1). Using a generalized linear model (glm) (using the 'glm()' function) this response was modeled to be an effect of the interaction between the five groups and the two developmental phases, namely the larval and pupal stage and the MC-ID as a random effect. The interaction between the day of the experiment with the other fixed effects were excluded as the models failed to converge. Next, any DPE-values lower than 1 (not a full day over 25 °C) were analysed. Two further generalized additive (gam) models (using the 'gam()' function from the 'mgcv'-package; (Wood, 2017)) were made, one for the larval phase and one for the pupal phase. Two models were chosen because the binomial model revealed that the DPE responded significantly differently for the five groups in the two different development phases. The model for the larval phase had the day of the experiment as a smoothed term, with an interaction with the group, and the MC-ID defined as a random slope within the groups. The model was weighted by the available sample size and used a beta distribution with the restricted maximum likelihood method of estimation. The difference in the model on the pupal phase was that it used the transformed DPE (square root of (1-DPE)) as the response variable, with a normal distribution and did not use weights or random effects.

Further main analysis focused on the effect of resource limitation and pesticide treatment on MCs using three readouts. (1) Mean DPE values of a MC over (a) the whole brood development phase (from first eggs to first drone eclosion or last full day (34) of experiment), (b) the larval development phase (from first eggs to first pupa or end of experiment), and (c) the pupal development phase (from first pupa to first male eclosion or end of experiment, excluding MCs that never had any pupae). (2) Total brood development duration (excluding MCs where no drones eclosed). (3) Probability of producing drones and total numbers of drones produced by a MC within the 35-day timespan. Additionally, we analysed the DPE-values cumulated over the days of development as a measure for total thermal investment into the brood and the weight of the live brood that was still present at the point of MC-termination.

These readouts served as response variables in generalized mixed models (glms). For each response variable, we began by creating an extensive glm including random effects (number of replaced workers and mean worker body size per MC) and several fixed effects (larval and pupal mean DPE and development duration, where applicable). To account for the fact that the *ad libitum* group had a significantly smaller sample size, a "weights"-term was included addressing this. Extended models were tested against simpler models to estimate the significance of specific terms. The models were tested against each other using the chi-square test in the 'anova()' function, as well as the 'AIC()' function, from the 'stats'-base-package. Factors that did not contribute to the fit of the model or decreased the quality, were dropped from the model. Where AIC-values between models did not significantly differ, the simpler model was chosen (Burnham and Anderson, 2004). This resulted in models including only the five groups as a fixed effect, because the other tested factors did not add to the fit of the model. For analyses on the pupal phase and the total development time, MCs that never had any pupae or did not produce any drones respectively were excluded. Pairwise post-hoc analyses between the treatment groups were conducted with the "emmeans"-function from the package of the same name (V. Lenth and Russel, 2023), adjusting the *p*-values with the "Tukey"-method to account for multiple comparisons. The "DHARMa"-package was used to test the fit of the models (Hartig, 2022).

In order to separately assess the interaction between GLY and FPF in the combinatorial treatment (GF), we calculated the predicted additive effect of the combinatorial treatment on the regarding readout, which is equal to the sum of the effects in the single treatment groups G and F (Jackson et al., 2016; Siviter et al., 2021). Comparing the observed effect in the GF group with the predicted additive effect allowed us to calculate the interaction effect size Hedges' d (standardized mean difference) and the 95 %-confidence intervals (Jackson et al., 2016; Siviter et al., 2021).

All statistical and graphical analyses were done in R version 4.0.2 (2020-06-22, (R Core Team, 2020)).

3. Results

3.1. Survival

Across all groups, only a few workers died per MC during the >35 days of testing. In one of five *ad libitum* MCs one worker died, in eight of seventeen Control MCs 1–4 workers died, in six of twelve MCs of the G-treatment 1–2 workers died, in five of twelve MCs of the F-treatment 1–2 workers died and in eight of twelve MCs of the GF-treatment 1–2 workers died. Groups did not differ in probability of worker mortality ($X^2 = 1.28$, df = 4, $p = 0.86$). Neither the feeding regime nor pesticide treatment affected the total number of dead workers per MC (feeding regime: $X^2 = 2.04$, df = 4, $p = 0.73$; pesticide treatment: $X^2 = 12.69$, df = 16, $p = 0.69$).

3.2. Brood temperature

First, we compared the ability of MCs in the different groups (*ad libitum* group and resource limited pesticide treatment groups: Control, G, F, GF) to maintain their brood at elevated temperatures throughout a whole day.

We fitted a logistic mixed model (estimated using ML and BOBYQA optimizer) to predict the probability of colonies maintaining their brood at elevated temperatures throughout the day with the interaction between developmental phase and treatment as fixed effects. We found that MCs in the Control and G-treatment in the larval phase did not differ from the *ad libitum* group (Fig. 2A). However, MCs of the F-treatment and the GF-treatment in the larval phase were significantly less likely to maintain the temperature above 25 °C than MCs in the *ad libitum* group (3.17-times (F) and 5.29-times (GF) lower likelihood) or in the Control MCs (4.25-times (F) and 6.92- times (GF) lower likelihood. Table S3). The effect of resource limitation is starkly visible at the pupal stage, as all four resource limited treatment groups showed significantly lower likelihoods of maintaining an elevated brood temperature throughout the day as compared to the *ad libitum* group. MCs of the *ad libitum* group were >25- times (range between 25- to 55- times; Table S3) likely to maintain elevated brood temperatures over 24 h compared to MCs from resource limited groups. However, there were no differences between MCs from the four different resource limited groups themselves.

Next, we analysed mean DPE values over the whole brood. Mean DPE values over the whole development were significantly lower in MCs of the F-treatment ($p < 0.01$; ~ −12 %; Fig. 2B; Table S3) and in the GF-treatment ($p = 0.05$; ~ − 9 %; Fig. 2B; Table S3) than in MCs of the Control. The MCs of the G-treatment did not differ from the Control ($p = 0.74$; Fig. 2B; Table S3). Compared to MCs of the G-treatment, MCs of the F-treatment ($p < 0.001$; ~ −15 %; Fig. 2B; Table S3) and the GF-treatment ($p = 0.01$; ~ −12 %; Fig. 2B; Table S3) had on average also significantly lower DPE values. MCs of the F-treatment and the GF-treatment did not differ ($p = 0.90$; Fig. 2B; Table S3). Mean DPE were also significantly lower in MCs of the F-treatment ($p < 0.001$; ~ −24 %; Fig. 2B; Table S3) and those of the GF-treatment ($p < 0.01$; ~ − 21 %; Fig. 2B; Table S3) when compared to MCs of the *ad libitum* group.

The same pattern is evident when looking at mean DPE-values only during the larval brood phase Fig. 2C). In the F-treatment and the GF-treatment, MCs had on average significantly lower DPE values than in

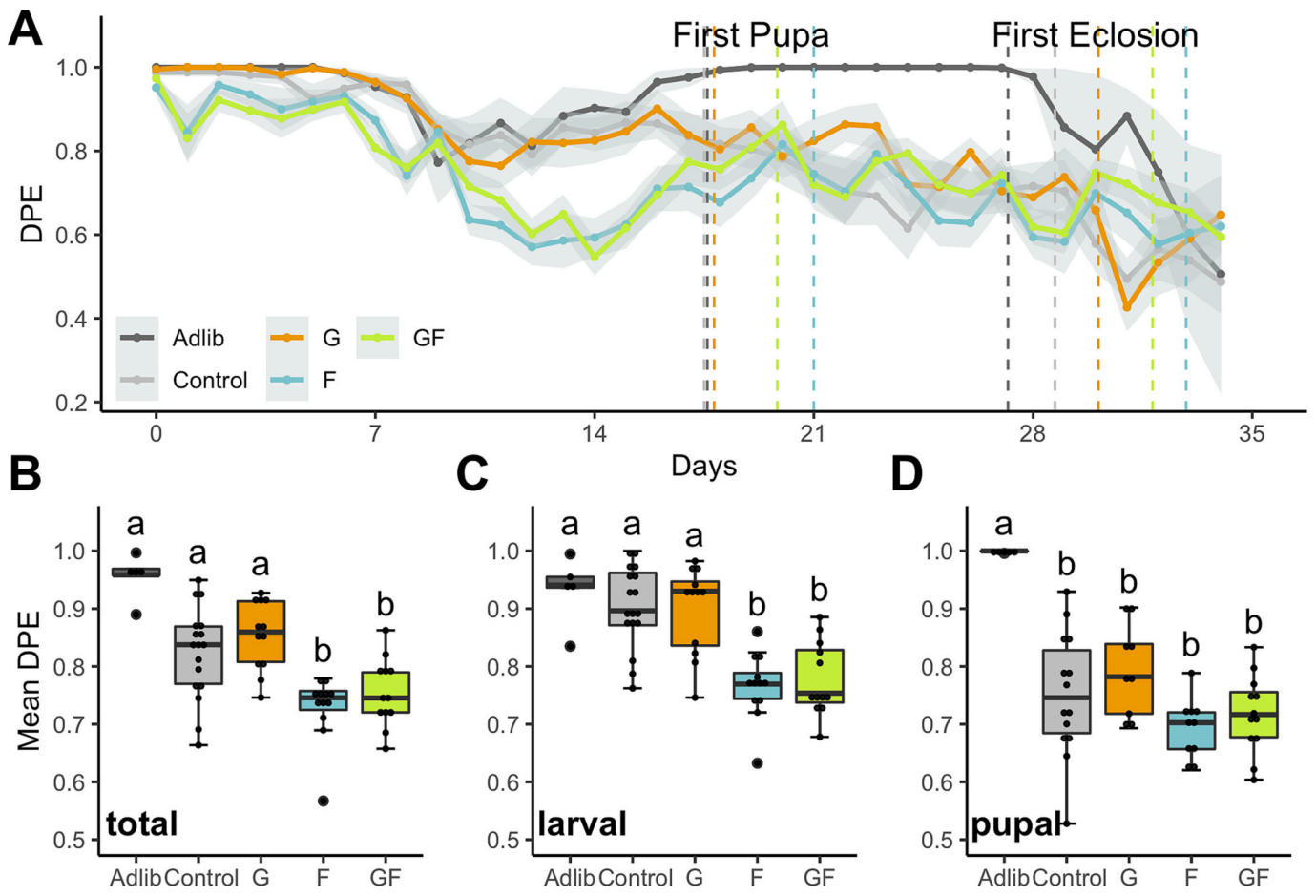

Fig. 2. Brood temperature in microcolonies; shown as DPE (daily proportion of elevated brood temperature (>25 °C)). Groups: *Ad lib: ad libitum* feeding regime; Control: sugar water limitation, grey; G: GLY treatment & sugar water limitation, orange; F: FPF treatment & sugar water limitation, blue; GF: combinatorial treatment & sugar water limitation, green. A: Mean DPE from day 0 to day 34 per group (day 0: first oviposition in a MC). Grey shading: SE. Vertical dashed lines: average day of first pupation and first drone emergence per group (*Ad lib*: $N = 5$; Control: $N = 17$; G: $N = 12$; F: N = 12; GF: N = 12). MCs of the *ad lib* group were more likely to maintain brood temp. Above 25 °C for a full day than MCs od any of the resource limited groups ($p < 0.001$). B-D: Mean DPE values in MCs of the five groups. Black dots: data from single MCs (mean DPE values for corresponding period per MC). Different letters indicate statistically significant differences (glm and post-hoc pairwise comparison, $p \leq 0.05$, Tukey-adjusted). B: Mean DPE over the total brood phase documented (first oviposition to first male or termination of MC, *Ad lib:* N = 5; Control: N = 17; G: N = 12; F: N = 12; GF: N = 12). Mean DPE-values were significantly lower in MCs of the F-treatment ($p = 0.0029$) and the GF-treatment ($p = 0.0509$) than in Control MCs. C: Mean DPE during the larval phase (first eggs to first pupation or termination, *Ad lib:* N = 5; Control: N = 17; G: N = 12; F: N = 12; GF: N = 12). Significantly lower DPE values in F and GF MCs than the Control (F: $p < 0.0001$; GF: $p = 0.0001$). D: Mean DPE during the pupal phase (first pupation to first male or termination). Only MCs that had any pupa at any point of the experiment were included in this analysis (*Ad lib:* N = 5; Control: $N = 16$; G: $N = 11$; F: N = 12; GF: N = 12; MCs that never had any pupae were excluded). Mean DPE values did not differ among the resource limited treatment groups but were significantly higher for the *ad libitum* group.

the Control (F: $p < 0.001$; ~ −15 %; GF: p < 0.001; ~ − 14 %; Fig. 2C; Table S3). Average larval DPE values also differed significantly between MCs of the G-treatment and those of the F-treatment (p < 0.001; ~ −15 %; Fig. 2C; Table S3) and of the GF-treatment (p < 0.001; ~ −14 %; Fig. 2C; Table S3). MCs in the G-treatment did not differ from Control MCs ($p = 1.00$; Fig. 2C; Table S3) and there was no difference between MCs of the F-treatment and the GF-treatment ($p = 0.99$; Fig. 2C; Table S3). DPE values during the larval phase were again significantly lower in MCs from the F-treatment ($p = 0.02$; Fig. 2C; Table S3) and the GF-treatment ($p = 0.04$; Fig. 2C; Table S3) than in the *ad libitum* MCs.

During the pupal phase mean DPE values were significantly lower in the MCs under resource limitation compared to the *ad libitum* MCs (*ad lib* – control: $p = 0.001$, ~ −29 %; *ad lib* – G: $p = 0.03$, ~ −21 %; *ad lib* – F: p < 0.001, ~ - 33 %; *ad lib* – GF: $p < 0.01$, ~ −28 %; Fig. 2D; Table S3). Mean DPE values during the pupal phase did not differ from the Control in any of the pesticide treatment groups.

3.3. Brood developmental time

Total brood development times were significantly longer in MCs of the F-treatment (32.89 ± 1.54 days; p = 0.001; Fig. 3A; Table S3) and the GF-treatment (31.82 ± 1.60 days; p = 0.01; Fig. 3A; Table S3) than in MCs of the Control (28.70 ± 2.67 days; Fig. 3A; Table S3). MCs of the G-treatment did not differ from those in the Control (30.09 ± 3.21 days; $p = 0.58$; Fig. 3A; Table S3). Total brood development time was shortest for the *ad libitum* MCs (27.20 ± 1.30 days; Fig. 3A).

3.4. Total thermal investment necessary for development

Across all groups, brood required the same total thermal investment to develop from egg to adult. We analysed the total proportion of time during which brood temperature was elevated (accumulated DPE values) from the appearance of the first egg to the emergence of the first drone. This measure of cumulative thermal investment did not differ between groups (tested using a glm, Fig. S2; Table S3.)

3.5. Reproductive output

Not all MCs successfully produced drones within the 35 days after first oviposition (Fig. 3B). In the Control, 14 of 17 MC (82 %), in the G-treatment 11 of 12 MCs (92 %), in the F-treatment 9 of 12 MCs (75 %) and in the GF-treatment in 11 of 12 MCs (92 %) produced drones. In the *ad libitum* MCs 5 of 5 (100 %) produced drones. Differences between groups were not significant ($X^2 = 0.21$, df = 4, p = 0.99).

3.6. Number of drones

Control MCs produced on average 4.41 ± 2.50 drones (Fig. 3B). MCs in the F-treatment (1.67 ± 1.50 drones; p = 0.001; Fig. 3B; Table S3) and in the GF-treatment (2.42 ± 1.24 drones; $p = 0.05$; Fig. 3B; Table S3) produced significantly less drones. MCs of the G-treatment did not differ from the Control in their reproductive output (3.92 ± 2.11 drones; $p = 0.97$; Fig. 3B; Table S3). The *ad libitum* MCs produced 6.6 ± 0.89 drones on average which was not different from the Control (Fig. 3B; Table S3).

3.7. Brood at time of colony termination

Brood weight per MC was twice as high in MCs of the F- and GF-treatment than in MCs of the Control (F: p < 0.001, +1.56 g; GF: p < 0.001, +1.79 g; Fig. S3A; Table S3) or of the G-treatment (F: p < 0.01, +1.45 g; GF: p < 0.001, +1.67 g; Fig. S3A; Table S3). Brood of all stages was found in all groups (Fig. S3B). The most brood was left in MCs of the *ad libitum* group.

A significant finding during brood dissection was that a total of 13 dead pupae were found exclusively in the brood of FPF-treated MCs. They all had a pupal cocoon and were in the first pupal stage (still larvae shaped). In some cases, it was not possible to clearly identify the pupal stage as they had degraded to brownish slime. Such dead pupae were found in seven of the MCs in the F-treatment group and in two of the MCs in the GF-treatment group, but in none of the MCs of the other groups.

3.8. Combinatorial effect

The Interaction effect size Hedges' d, a comparison between the observed effect and the predicted additive effect of FPF and GLY was calculated for the effect of the combinatorial GF-treatment for all of the readouts presented. Potentially additive, synergistic and antagonistic effects were detected for the different measurements (Fig. S4; Table S4).

4. Discussion

Our study provides evidence for a sublethal but highly critical effect of flupyradifurone (FPF) on resource limited bumblebee microcolonies. We show that long term oral exposure reduces the collective ability to

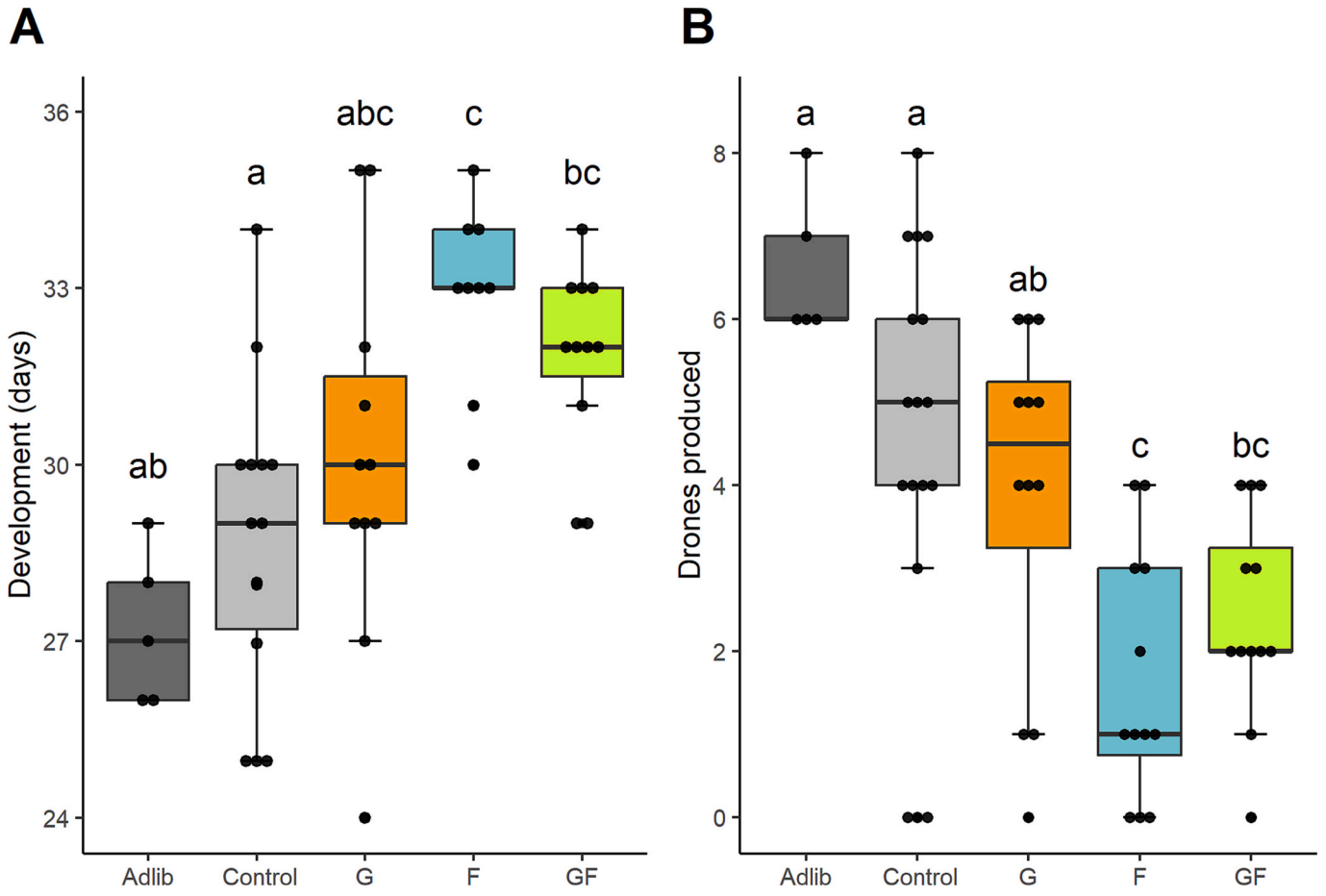

Fig. 3. Developmental time and reproductive output in microcolonies. A: Development time (first oviposition to first eclosion; *Ad lib:* N = 5; Control: *N = 14*; G: N = 11; F: *N* = 9; GF: N = 11). MCs in which no drones eclosed are excluded. Compared to the Control, development was significantly prolonged in the F-treatment ($p = 0.0012$) and in the GF-treatment ($p = 0.0146$). B: Number of drones eclosed per MC (*Ad lib:* N = 5; Control: N = 17; G: N = 12; F: N = 12; GF: N = 12). MCs in the Control produced significantly more drones in the given time than those in the F-treatment (p = 0,0043) and those in the GF-treatment ($p = 0.0104$). Groups: *Ad lib: ad libitum* feeding regime; Control: sugar water limitation, grey; G: GLY treatment & sugar water limitation, orange; F: FPF treatment & sugar water limitation, blue; GF: combinatorial treatment & sugar water limitation, green. Black dots: data from single MCs. Different letters indicate statistically significant differences (glm and post-hoc pairwise comparison, p ≤ 0.05, Tukey-adjusted).

raise brood, with a decrease in reproductive output by over 50 % compared to control colonies. Such a large impairment in colony growth is bound to directly impact colony fitness. (Owen et al., 1980; Schmid-Hempel and Schmid-Hempel, 1998).

Our data shows clearly that a certain amount of cumulative thermal energy is necessary for successful development from egg to adult in bumblebee brood. Temperature is the main driver in insect development (Cartar and Dill, 1991; Goulson, 2013; Grad and Gradišek, 2018; Groh et al., 2004; Wigglesworth, 2012) and development of bumblebee brood is fast and survival is high only within a narrow temperature window of 28 °C–35 °C (Weidenmüller et al., 2022). Times of elevated brood temperature in our MCs therefore directly predict brood developmental time - the more seamlessly the necessary total thermal energy was delivered (the higher the DPE), the faster the brood developed. Any disruption of this process, *i.e.* periods of no incubation and reduced brood temperature, directly impact brood development, colony growth and ultimately colony fitness, especially when ambient temperature is low (Weidenmüller et al., 2022), *e.g.* in bumblebee colonies in early spring, and when colonies are still small. Brood temperatures in our experiment fell to ambient room temperature of around 22 °C when not incubated, and brood development essentially ceased during these periods, corresponding to findings by Cartar and Dill (1991) and Weidenmüller et al. (2022). We suggest that the significant reduction in reproductive output documented in MCs exposed to FPF resulted directly from a decrease in thermal energy invested into their brood. Throughout the whole experimental time, these MCs showed a significant impairment in their ability to maintain their brood at elevated temperatures: their daily proportion of elevated brood temperature (DPE) was significantly lower compared to Control MCs, especially during the larval brood phase. This impairment had the expected knock-on effect of prolonged brood developmental times and consequently on reproductive output and colony growth.

MCs were provided fresh, untreated pollen bread daily. While we cannot exclude that some of the treated sugar water entered larval food, we are confident that the documented effects of extended brood development and decreased reproductive output are causally related to the decrease in thermal investment documented in our data. Treatment did not affect the total amount of thermal investment necessary for brood development; and brood production generally was not affected by pesticide exposure. The finding that when MCs were terminated, there was more brood still in the "pipeline" in the FPF treated MCs compared to non FPF exposed MCs supports the hypothesis that brood was moving along slower on the 'developmental conveyer belt' in FPF exposed MCs due to a decrease in brood temperatures rather than FPF causing lesser brood production. The large number of dead, freshly pupated larvae found exclusively in the FPF treatment group further underlines the troubling effect of this pesticide. It remains to be investigated if this developmental phase is especially susceptible to temperature fluctuations or if there are direct toxic effects of FPF exposure during larval development leading to this phenomenon.

How FPF disrupts the ability to maintain high brood temperatures remains to be explored. Mechanisms underlying thermogenesis or energy utilization in general may be affected. Studies in honeybees suggest an increase in energy requirement connected to neonicotinoids, possibly connected to detoxification and resulting in compensatory sugar water uptake (Tosi et al., 2017). In bumblebees compensatory sugar water uptake has also been documented following an immune challenge (Moret and Schmid-Hempel, 2000; Tyler et al., 2006) or following GLY exposure (Weidenmüller et al., 2022). We did not record how long sugar water was available per microcolony following feeding, and thus have no direct evidence for compensatory sugar water uptake in FPF exposed MCs. However, sugar water was always used up, and brood temperatures in our MCs reliably showed a stable plateau of elevated temperatures (> 25 °C) following feeding, before crashing to room temperature and remaining low until the next feeding event.

The duration of these stable plateaus was significantly shorter for MCs treated with FPF (data not shown) which leads us to assume a faster use of the sugar water supplies in these MCs and therefore earlier energy shortage and brood temperature decline. A recent study showed that even low concentrations of FPF (10μg/l) perturb the gut microbiome of bumblebees, resulting in a downregulation of the carbohydrate metabolism (Zhang et al., 2022). Additionally, FPF was documented to lower feeding motivation in bumblebees (Siviter and Muth, 2022).

If FPF indeed affects thermogenesis in general, this would have ramifications not only into collective brood rearing but also into the ability of foragers to fly out under cooler conditions, which would additionally hamper colony growth and survival chances. A further explanation for lower brood temperatures in FPF exposed MCs could be a general impairment of workers resulting in reduced activity and withdrawal from the nest as described in Crall et al. (2018) for bumblebees following imidacloprid exposure. However, we did not observe any withdrawal from the nest or inactivity in workers during our daily inspections.

We found no significant effect of GLY exposure on the parameters measured in the microcolonies in our study. While there is an increase in developmental time and a decrease in reproductive output in GLY-exposed MCs, these effects are non-significant. Straw and Brown (2021a) also report no effect on reproductive output of GLY-treated microcolonies (fed *ad libitum*). This is in contrast to a recent study on queenright bumblebee colonies, showing a clear impact of long-term GLY exposure on collective thermoregulatory ability (Weidenmüller et al., 2022) when colonies experience a resource shortage. One possible explanation for this discrepancy is that GLY effects may be mediated largely *via* gut dysbiosis (Motta et al., 2018; Motta and Moran, 2020). If this is the main mechanism, exposure to GLY during larval development or during the first hours as an adult, when individual gut microbiome is formed *via* social transmission (Koch and Schmid-Hempel, 2011), may be especially impactful later in adult life. Workers in our microcolonies were first exposed to GLY when they were already a few days old. GLY may also impact the social structure in additive ways that become apparent only in larger group sizes or in queenright colonies. Future studies should further dissect the mechanisms of GLY and the life stages and sensory abilities that are especially impacted by GLY exposure.

We found no clear interactions between FPF and GLY in our combined treatment group. MCs of this treatment seem to be performing slightly better than the ones with the pure FPF treatment, yet significantly worse than the Control. Analysing the interaction effect size Hedges' d, yielded contradicting results for the different readouts. From an additive effect between FPF and GLY on the total mean DPE in a MC, to an antagonistic effect on total development and a synergistic effect on the number of drones produced. It may be that different traits react differently, showing more or less susceptibility to the same stressor. It is also possible that we failed to analyse the actual interaction effect between FPF and GLY, since the effect of GLY was so subtle. These results show that this possible interaction effect deserves further investigation. Previous work evidenced synergistic effects of FPF and a common fungicide (propiconazole) on honeybee survival and behaviour (Tosi and Nieh, 2019).

All MCs in our treatment groups were maintained under resource limitation. Colonies in the wild are regularly exposed to resource limitation bottlenecks (Biesmeijer et al., 2006; Goulson et al., 2015; Rotheray et al., 2017), especially and increasingly in agricultural landscapes (Samuelson et al., 2018), where they are also most likely to be exposed to pesticides. Classical laboratory conditions with *ad libitum* feeding of sugar water at high room temperatures and maximum colony growth rates are therefore highly unnatural. Several studies have shown that resource limitation and nutritional deprivation modulate susceptibility to pesticides (Tong et al., 2019; Weidenmüller et al., 2022). In our experiment, MCs with *ad libitum* sugar water kept their brood at constant elevated brood temperatures for most of the time except for a dip during the second week of brood development, that can be found in all the groups. We currently speculate this to be connected to different thermal

requirements of the brood during that developmental stage. Just as described in Cartar and Dill (1991), our MCs under resource limitation dropped to ambient temperatures under energy deprivation (*i.e.* ceasing brood incubation when the provided sugar water was used up; Fig. S1). There is thus a clear effect of resource limitation on brood development and colony growth *via* changes in collective brood temperature: Total development took up to eight days longer in resource limited Control MCs than in MCs with *ad libitum* access to sugar water and nearly constant elevated brood temperatures, and drone production was 33 % lower. This confirms the findings of Hemberger et al. (2020), where microcolonies being fed low food rations produced 27 % less drones. Colonies and individuals with *ad libitum* sugar water availability, as used in classical risk assessment testing and in most scientific studies, may be able to compensate physiological impairments *via* compensatory sugar water uptake. While we can only speculate about the effect of FPF in well fed colonies, testing colonies and microcolonies under resource limitation represents more natural and therefore more relevant conditions.

Using microcolonies in ecotoxicological studies has been recommended by numerous authors (Klinger et al., 2019; Tasei and Aupinel, 2008). Others have discussed this approach critically, arguing that the use of microcolonies has the risk of "erroneous" conclusions, as some sublethal effects might only be observed in free-foraging queenright field colonies and worker and gyne production is neglected (Van Oystaeyen et al., 2021). While we understand these concerns and the restrictions of the approach, microcolonies offer the benefit of documenting both individual and collective behaviour and sub-lethal alterations in behaviour under highly standardized conditions and with a high degree of genetic similarity between test colonies and can therefore provide a valuable first tier approach to investigating sublethal effects of a substance. Our study shows that using brood temperature as a collective readout in such microcolonies can serve as a highly informative and reliable behavioural metric when testing for sublethal effects of pesticides. We documented three parameters: brood temperature, brood development, and reproductive output; our findings demonstrate that documenting only a few days of brood temperature during the larval phase in microcolonies is a sufficient and reliable indicator of serious impairments in the critical process of collective brood care. Our results suggest that studies using microcolonies for high throughput risk assessment testing need to be aware that while strong effects will show up, more subtle effects may be missed. The significant effects on collective thermoregulation and reproductive output documented in microcolonies exposed to FPF in our lab study will, with high certainty, also show up in and impact whole colonies encountering FPF in the field. The effects of glyphosate in comparison are clearly more subtle and not significant in our experimental approach and at our sample size. While in our study, the effects of GLY on collective thermoregulation in microcolonies show up as weak, non-significant trends, a previous study using whole queenright colonies found them to be significant (Weidenmüller et al., 2022). Clearly, microcolonies are a helpful and important tool in investigating sublethal effects on the collective. Monitoring whole queenright colonies, although costly and complicated, due to often large inter-colony variability in worker size, colony growth rate, timing of sexual reproduction *etc.* (Duchateau et al., 2004; Spaethe and Weidenmüller, 2002), will however remain an important and sometimes necessary additional step.

Pesticides are ubiquitous, with over 4 billion tons applied worldwide in 2019 (FAOSTAT, 2021). The field realistic exposure of insects to agrochemicals remains contentious, and there is a paucity of data documenting actual exposure rates in different habitats. The concentrations used in our study are in the lower end of concentrations used in previous studies, yet similar and higher concentrations have been measured in the field. Recent studies indicate that flying insects are regularly exposed to a multitude of insecticides, herbicides and fungicides (Uhl and Brühl, 2019), with exposure occurring in various and potentially cumulative ways. For bumblebees, residues accumulate in nests (in the food stores and in the wax produced) where all nest mates and all brood stages can also be exposed. Given that FPF is marketed worldwide, our findings on its effects on bumblebees are concerning. While restrictions on neonicotinoids have been implemented following evidence of their adverse effects on beneficial pollinators, their recent successors may be no less harmful. Recently EFSA (European Food and Safety Authority) recommended further studies addressing the effects of chronic exposure of flupyradifurone on bees, yet mainly focusing on mortality and on honeybees (EFSA et al., 2022). Classical risk assessment testing concluded FPF to be relatively bee safe. Risk assessments usually only test the lethality of a novel active ingredient (EFSA et al., 2010). It is worth noting that several studies showed co-formulants of the final product to have adverse effects themselves or to increase the effect of the active ingredient (Straw et al., 2021, 2022; Straw and Brown, 2021b). Although our study focused on the active ingredient as well, the approach we presented is easily applicable to test the effects of the whole formulation of a pesticide product. It is notable that for a long-term exposure time of more than five weeks, we found no effects on individual survival; the pesticide concentrations used in our experiment would clearly easily pass regulatory risk assessment studies (Carleton, 2014) as non-toxic. We demonstrate how this approach to risk assessment underestimates the risks a pesticide may pose *via* significant non-lethal and behavioural effects.

Our study raises serious concerns about the ecotoxicological profile of FPF and its safety for wild bees. It should serve as a warning that even if effects are sub-lethal at the individual level, they may have devastating effects on colonies and populations, contributing to the many calls for improved risk assessment procedures (Straub et al., 2020). Finding reliable, relevant behavioural metrics will be key in our ability to assess the actual risks posed to our ecosystems by the heavy and ubiquitous use of agrochemicals.

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CRediT authorship contribution statement

LF and AW designed and conducted the experiment, LF and DR analysed the data, and LF and AW wrote the manuscript.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Data availability

Data will be made available on request.

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