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# Novel fungicide and neonicotinoid insecticide impair flight behavior in pollen foraging honey bees, *Apis mellifera*

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**Bees are often exposed to pesticides affecting physiological functions and molecular mechanisms. Studies showed a potential link between altered expression of energy metabolism related transcripts and increased homing flight time of foragers exposed to pesticides. In this study, we investigated the effects of thiamethoxam and pyraclostrobin on longevity, flight behavior, and expression of transcripts involved in endocrine regulation (*hbg-3*, *buffy*, *vitellogenin*) and energy metabolism (*cox5a*, *cox5b*, *cox17*) using radio frequency identification (RFID) technology and quantitative polymerase chain reaction. Parallel, a laboratory study was conducted investigating whether pesticide exposure alone without the influence of flight activity caused similar expression patterns as in the RFID experiment. No significant effect on survival, homing flight duration, or return rate of exposed bees was detected. The overall time foragers spent outside the hive was significantly reduced post-exposure. Irrespective of the treatment group, a correlation was observed between *cox5a*, *cox5b*, *cox17* and *hbg-3* expression and prolonged homing flight duration. Our results suggest that flight behavior can impact gene expression and exposure to pesticides adversely affects the expression of genes that are important for maintaining optimal flight capacity. Our laboratory-based experiment showed significantly altered expression levels of *cox5a*, *cox6c*, and *cox17*. However, further work is needed to identify transcriptional profiles responsible for prolonged homing flight duration.**

**Keywords** Agrochemicals, Pollution, Sustainability, Homing flight activity, Gene expression, Biomarker

The global intensification of industrial agriculture has led to an increased usage of plant protection products (PPPs) and has been identified as a key factor contributing to wild bee population declines and impaired health of managed honey bee[1]. Primarily used in the agricultural sector, but also in forestry, horticulture and home gardens, PPPs including herbicides, fungicides, insecticides, acaricides and plant growth regulators aim to protect plants and crops from natural pests, diseases, and weeds. Due to run-off and air-drift, PPPs not only remain on the treated fields[2] but can also contaminate wild flowers along the edges of crop fields causing unintentional risk to non-target species[3]. Currently among the most used PPPs globally are the neonicotinoids, including the compounds acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam[4]. Due to their systemic and hydrophilic properties, neonicotinoids are absorbed by plants and distributed to all parts of the plants, including nectar and pollen[5]. Therefore, bees are frequently exposed to a wide range of PPPs via contaminated pollen, nectar, guttation fluids, as well as water and soil[6,7]. By acting as agonists of the nicotinic acetylcholine receptors, neonicotinoids remain at the receptor and continuously transmit nerve impulses, leading to overexcitation of the insect nervous system and inducing systemic effects as they are not immediately hydrolyzed by acetylcholinesterase[8]. Subsequently, their mode of action can have severe neurotoxic effects causing paralysis and ultimately lead to the death of the target pests as well as non-target organisms[5]. Indeed,

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many studies have revealed both lethal as well as inadvertent sublethal effects of neonicotinoids on bees[9,10]. Of particular concern are studies showing that field relevant dosages of these substances can impair foraging and homing abilities[11], which are likely to have negative consequences for colony fitness[1,12]. These findings have stirred much debate and concern both in the scientific community and public[13], leading to the prohibition of all outdoor uses of three neonicotinoids in Europe in 2018[14]. Nevertheless, neonicotinoids remain registered for use on > 140 different crops in over 120 countries[15], with extensive agricultural application occurring in Latin America, North America and Asia[16].

Bees and other pollinating insects are however rarely exposed to only one specific xenobiotic, but rather to a mixture of substances simultaneously[17], including fungicidal PPPs[18]. Fungicides account for approximately 11% of the total global PPPs used[19]. Flowering crops (e.g., orchards and berries) are particularly attractive to honey bees and other pollinators, yet they are often treated with fungicides which inevitably increases the likelihood of exposure[20]. Fungicides from the strobilurin class, such as pyraclostrobin, inhibit cellular mitochondrial respiration of fungi. They bind to the quinol oxidation ($Q_0$) complex III (bc1 complex), which leads to an interruption of ATP production[21]. Field-realistic concentrations of such fungicides have been shown to elicit lethal effects[22] as well as cause sublethal negative effects on honey bees[23,24] and wild bees[6], in particular when they interact with other common PPPs[18,22]. For instance, honey bee workers from colonies simultaneously exposed to PPPs revealed impaired brood rearing capacities, reduced flight abilities as well as increased pathogen susceptibility - all of which are argued to increase the likelihood of colony failure.

In addition to the negative physiological effects on bees, there is evidence that neonicotinoids and fungicides can have effects at the molecular level. Exposure of honey bees to sublethal concentrations of neonicotinoids and fungicides altered the expression of various transcripts in the brain, such as acetylcholine receptors, vitellogenin, immune system transcripts and transcripts linked to energy metabolism[25,26]. By analyzing global gene expression in the brain of honey bees after exposure to environmental realistic concentrations of different neonicotinoids, changed expression of genes linked to metabolism, detoxification and oxidative phosphorylation was found[27,28]. Similar results were obtained when analyzing transcripts of oxidative phosphorylation after neonicotinoid exposure by applying quantitative PCR[29]. Further, a study using radio frequency identification (RFID) technology revealed a potential link between transcriptional changes and flight behavior after exposure to the neonicotinoid thiacloprid[29]. The underlying reasons for these observations remain elusive and may be due to disturbed orientation abilities or altered energy metabolism of the foraging bees.

To shed light on how the expression of transcripts related to energy metabolism and the endocrine system relate with flight behavior in honey bees, we conducted a RFID experiment which enabled us to assess the lethal (i.e., longevity) and sublethal effects (i.e., homing flight duration, return rate, out time, and gene expression) of PPP exposure on foraging workers. This study aims at analyzing the lethal (i.e., longevity) and sublethal effects (i.e., homing flight duration, return rate, out time) of thiamethoxam and pyraclostrobin on honey bees, the effects of both pesticides on the expression of transcripts related to energy metabolism and the endocrine system and to identify a possible link between gene expression and flight behavior and based on that, to identify potential biomarker transcripts.

We hypothesized that pesticide exposure alters gene expression related to energy metabolism and endocrine function in honey bees, affecting their flight behavior. To test this, we conducted a RFID experiment to assess both lethal (longevity) and sublethal (homing flight duration, return rate, out time, and gene expression) effects of PPP exposure on foraging workers. Additionally, we performed a laboratory study where foragers were exposed to thiamethoxam or pyraclostrobin, and gene expression was analyzed without performing homing flight. By comparing gene expression patterns between these two conditions, we aimed to determine whether pesticide exposure alone could predict changes in flight behavior. This study seeks to identify predictive biomarkers for homing and flight behavior disruptions caused by PPP exposure.

## Results

### Control (C) vs. acetone solvent control (SC): survival, returning rate and out-hive time

No significant difference was observed between control and solvent control cumulative survival ($z = 0.54$; $p = 0.589$; Fig. S5A), where the average survival for workers used for the RFID test was 5.2 ± 0.4 days (mean ± SE). Survival did significantly differ amongst the three colonies ($z = 2.45$; $p = 0.014$). No significant difference in return rate was observed ($z = -1.91$; $p = 0.056$; Fig. S5B), where the return rate across both treatments was 77.48% ± 0.3 (mean ± SE [%]). A significant difference between control (75.24 ± 2.86) and solvent control (106.08 ± 4.94) treated bees was observed for the out-hive time ($z = 5.93$; $p < 0.001$; Fig. S5C), resulting in a 41% difference in spending less time outside their hive (mean ± SE). Likewise, colony as well as the day had a significant effect on the duration spent outside the hive (both $z$'s > 2.87, $p$'s < 0.004). To ensure a fair comparison of the potential effects of the used pesticides, we opted to excluding the control treatment from the following analyses based on the observed effect of acetone (solvent control) on the out-hive time of the bees. The following statistical analyses compared treatment effects with acetone solvent control group.

### Treatment survival time, return rate, homing flight duration and out-hive time

Pollen foragers labeled for longevity assessment, including foragers that returned beyond two hours, were further monitored for survival, return rate and out-hive time (C: $n = 55$, SC: $n = 51$, TMX: $n = 39$, PS 2.43 µg/bee: $n = 49$, PS 5.98 µg/bee: $n = 47$, Table S4). No significant difference in longevity was observed among the treatment groups ($\chi^2 = 33.38$; $p > 0.69$); where mean survival time post-exposure across all treatments was 5 ± 0.29 [d] (mean ± SE; Fig. 1A). Likewise, neither worker age at exposure nor colony revealed significant effects (both $z$'s > − 0.10; both $p$'s > 0.33). Return rate did not significantly differ amongst the treatment groups ($z$ = -0.62, $p = 0.536$; Fig. 1B); where the average return rate across all treatments and runs was 65.9% ± 0.23 (mean ± SE [%]). Likewise, colony revealed no significant effect on return rate ($z = -0.75$, $p = 0.451$). No significant effects

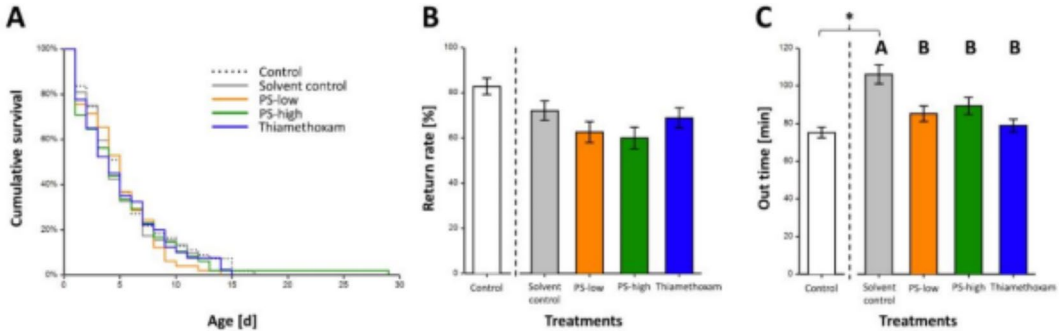


**Fig. 1**. Survival, return rate and out time (i.e., time spent outside of hive) of pollen foragers in the RFID test. Pollen foraging honey bees were either exposed to sucrose solution only (control), 5% acetone control (solvent control), pyraclostrobin at dose of 2.43 µg/bee (PS-low) or 5.98 µg/bee (PS-high) and thiamethoxam at dose of 1 ng/bee. (**A**) Cumulative survival of foragers across all treatment groups revealed no significant difference ($N = 244$, $\chi^2 = 33.38$; $p > 0.69$). (**B**) Return rate did not significantly differ between control and solvent control (N = 222; $\chi^2 = 3.86$, $z = -1.91$; $p = 0.056$). Likewise, the rate of pollen foragers returning within 24 h post-exposure revealed no statistical differences amongst treatment groups ($N = 437$, $\chi^2 = 0.84$; $p > 0.62$) and solvent control. (**C**) A significant difference in out time [min] was observed between control and solvent control ($N = 830$, $\chi^2 = 46.48$, $z = 5.93$; $p < 0.001$). Furthermore, forager bees exposed to pesticides revealed a significantly reduced out time compared to solvent control individuals ($N = 1317$, $\chi^2 = 36.08$, $z = -5.11$; $p < 0.001$).

were detected for homing flight duration (return time) ($p > 0.63$). No significant difference was observed across the pesticide exposed treatment groups (*bmct*; $z < 0.82$; $p > 0.68$; Fig. S7). The median homing flight duration of exposed and non-exposed individuals from the three different days and colonies are enclosed in supplementary Fig. S6 and Table S6.

Irrespective of the substance, pesticide exposure revealed a significant negative effect on out-hive time [h] ($z$ = -5.11; $p < 0.001$, resulting in exposed individuals (84.66 ± 2.34) spending 25% less time outside of their hives across their lifespan compared to the solvent control (106.08 ± 4.94; mean ± SE [h]; Fig. 1C). Fig. S7). Lastly, both day and colonies had a significant effect on out-hive time (both $z$'s > 3.83; $p < 0.001$, Table S7).

## Gene expression analysis of foragers performing homing flight

The brains of foragers that returned within two hours of release were analyzed for gene expression (C: $n = 36$, SC: $n = 29$, TMX $n = 34$, PS 2.43 µg/bee: $n = 22$, PS 5.98 µg/bee: $n = 18$, Table S3). The exposure to thiamethoxam or pyraclostrobin revealed no significant effect on energy metabolism transcripts (Fig. S8, Table S8) or endocrine transcripts (Fig. S9, TableS9), yet an increased variation between different genes was observed. We also evaluated whether there is a link between the gene expression and the homing flight duration (return time) and performed a correlation analysis. The result revealed a significant positive correlation between homing flight duration and the expression level of *cox5a*, *cox5b*, *cox17* and *hbg-3* (all $z$'s > 3.89; all $p < 0.001$) (Fig. 2). The expression rate of *vitellogenin* and *buffy* did not significantly correlate with homing flight duration (both $z$'s = 0.13; both $p$'s > 0.86).

## Gene expression analysis of foragers without performance of homing flight

The expression of *cox5a* was significantly inhibited by exposure to 2.43 µg/bee pyraclostrobin ($p = 0.043$; Fig. 3). Likewise, the expression of *cox6c* post-exposure to both pyraclostrobin concentrations was significantly inhibited ($p = 0.042$; $p > 0.026$; Fig. 3). Also significantly induced was the expression of *cox17* after exposure to thiamethoxam ($p < 0.001$; Fig. 3; Table S10). The exposure to thiamethoxam or pyraclostrobin did not significantly alter gene expression of the endocrine transcripts *vitellogenin*, *hbg-3* and *buffy* (all $p$-values > 0.05; Fig. S10, TableS11).

A visual comparison of gene expression pattern between RFID experiment and laboratory study is shown in Fig. 4. The expression patterns between the two approaches were different. Exposure to thiamethoxam strongly induced *cox5a* and *cox17* expression in the laboratory study whereas this was not the case in the RFID experiment. Pyraclostrobin lead to an up-regulation of *cox5b* in the RFID experiment, yet this was not the case in the laboratory study. Expression levels of *vitellogenin* were induced post-exposure to thiamethoxam and pyraclostrobin in the laboratory study yet the contrary was observed in the RFID study.

## Discussion

Here we show that acute oral exposure of foragers to pyraclostrobin or thiamethoxam did not significantly affect their survival, homing return rate or homing flight duration, whereas the time spent outside the hive (i.e., out time) was significantly reduced. In contrast, data from Christen et al.[29] revealed a significant reduction in return rate after 1 ng thiamethoxam/bee exposure. Reasons for the observed disparity may be due to the varying age of foragers. In the present study, foragers were all from the same age cohort (i.e., 20 days ± 1 day) whereas in the previous study they were randomly selected at unknown ages. It has been shown that increasing age can negatively influence flight performance[30] and increase susceptibility towards xenobiotic exposure[31].

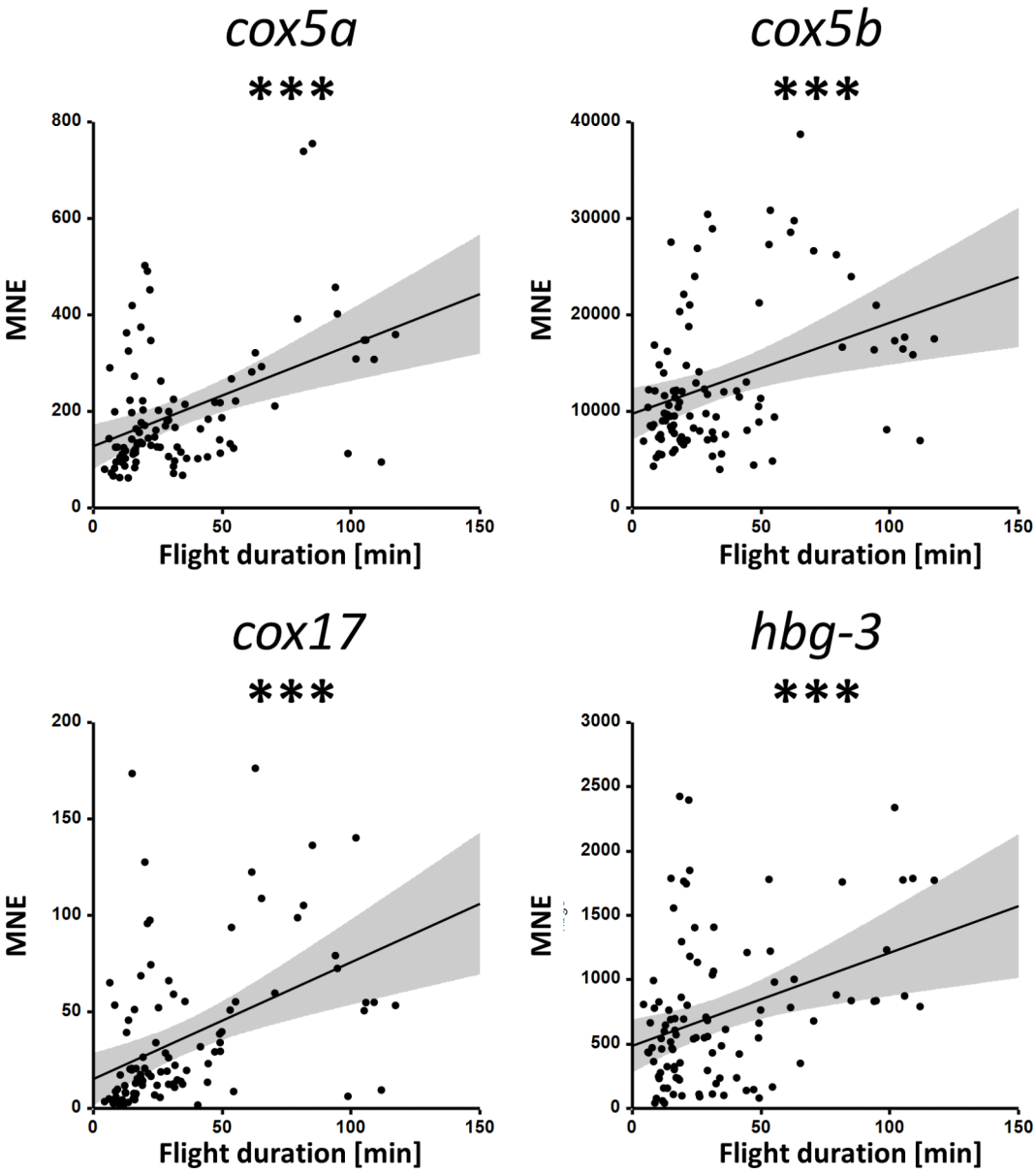


**Fig. 2**. Correlation of *cox5a*, *cox5b*, *cox17* and *hbg-3* gene expression in the brain of pollen foraging honey bees and homing flight duration. A significant positive correlation was observed for all four genes (*** = $p < 0.001$).

Our data highlights the importance of including both negative controls (i.e., sugar water only) and solvent controls to ensure robust ecotoxicological risk assessments when testing chemical substances. Studies on the acute toxicity of acetone for insects are contradictory and potential side effects of acetone on behavior traits as well as molecular mechanisms of bees are understudied. A study analyzing the toxic effects of acetone on stored-product insect pests, such as moths, showed no toxicity by topical contact[32], while exposure to high concentrations of acetone vapor led to an increased mortality[32,33]. Our study revealed no such negative effects of acetone exposure on the survival of bees. Due to the significant effect at the sublethal level, all pesticide exposure effects were compared to the solvent control (5% acetone) to ensure reliable comparability.

Our data suggest that the tested dosages of pyraclostrobin and thiamethoxam had no significant effect on the homing ability of foragers. However, under natural conditions, foragers most likely experience simultaneous exposure to a wide range of PPPs over several days - if not weeks - due to overlapping crop blooming periods[34], contact with dust emitted during drilling of PPP treated seeds[35] or crop and non-agricultural foraging areas being contaminated because of water run-off[17,36]. A study demonstrated that the fungicide PristineR® with the active ingredients boscalid (25%) and pyraclostrobin (13%) revealed a severe impact on honey bee cognition[37]. These findings highlight that additional research is needed to better understand the mode of action of pesticides on bees.

This study is the first to document the long-term effects of acute pesticide exposure on individual forager longevity under field conditions. While our findings confirm that the applied concentrations are non-lethal,

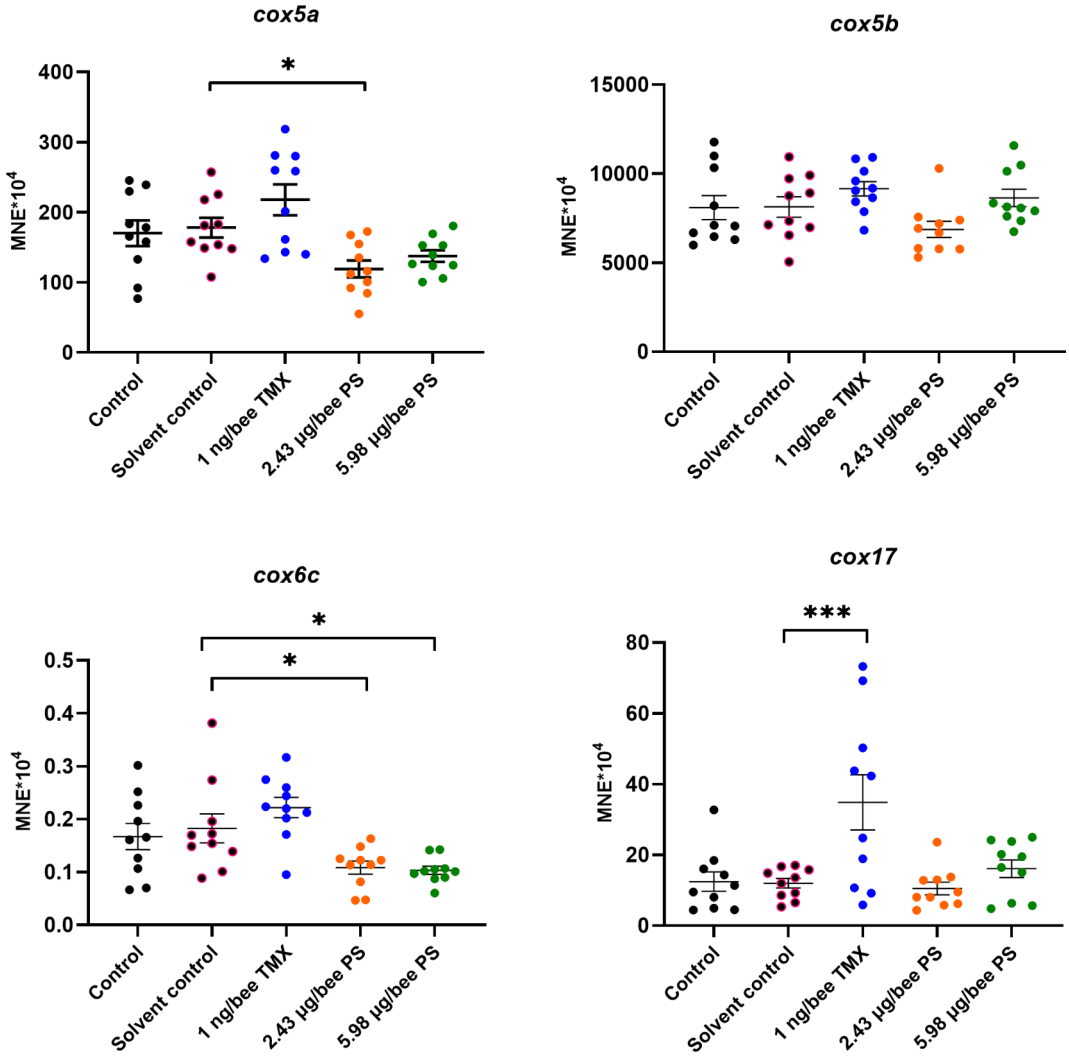


**Fig. 3**. Expression level of *cox5a*, *cox5b*, *cox6c* and *cox17* in the brain of exposed pollen foragers honey bees exposed under laboratory conditions. Single dots represent the expression level of individual pollen foragers ($n = 10$ bees per group). Pollen foragers were exposed either to 1 ng/bee thiamethoxam or pyraclostrobin at a concentration of 2.43 µg/bee or 5.98 µg/bee in single feeding without a subsequent flight phase. Significant differences between solvent control (5% acetone control) and exposed pollen foragers were marked with the following asterisks: $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

the potential impacts of chronic exposure to thiamethoxam and pyraclostrobin on survival require further investigation. Previous studies have shown that chronic sublethal exposure during larval development or adulthood can significantly reduce survival. Although acute exposure to field-relevant doses may not directly affect survival, our data suggest that sublethal effects are more common and could have serious implications for bee health.

In contrast, our data demonstrate that thiamethoxam and pyraclostrobin can negatively impact the time foragers spend outside the hive e.g. foraging. In contrast, we observed no significant effect on the return rate or flight duration of the foraging bees returning to the hive. Considering that the transcripts of energy metabolism were also significantly affected, this could indicate that pesticide exposure may have weakened them, hence e.g. performing shorter foraging trips. A recent study by Ma et al. 2024[38], demonstrated that the eastern honey bees, *Apis cerana*, exposed to a combination of three pesticides (imidacloprid, chlorpyrifos and glyphosate) showed significantly reduced flight duration and total flight distance in a flight mill experiment. Using transcriptomic data, Ma et al. (2024)[38] revealed that the combined exposure impaired the lipid metabolism and degradation of carbohydrate derivatives in test bees - both are important for energy production during flight. In addition, the upregulation of cytochrome P450 genes indicates an impaired detoxification capacity that may affect the immune response and energy metabolism of bees[38]. A study by Dosseli et al. 2016[39] showed that bees infected with the microsporidia *Nosema* performed short yet more frequent flights than non-infected bees. The reallocation of energy to the immune defense, which is known to be energetically costly[40], likely comes at the expense of altered flight behavior. In our experiment, bees exposed to pesticides may have adapted their flight activity to save energy and increase their chances of survival[41]. Similarly, Tosi et al.[42] also reported an excitatory effect respectively a

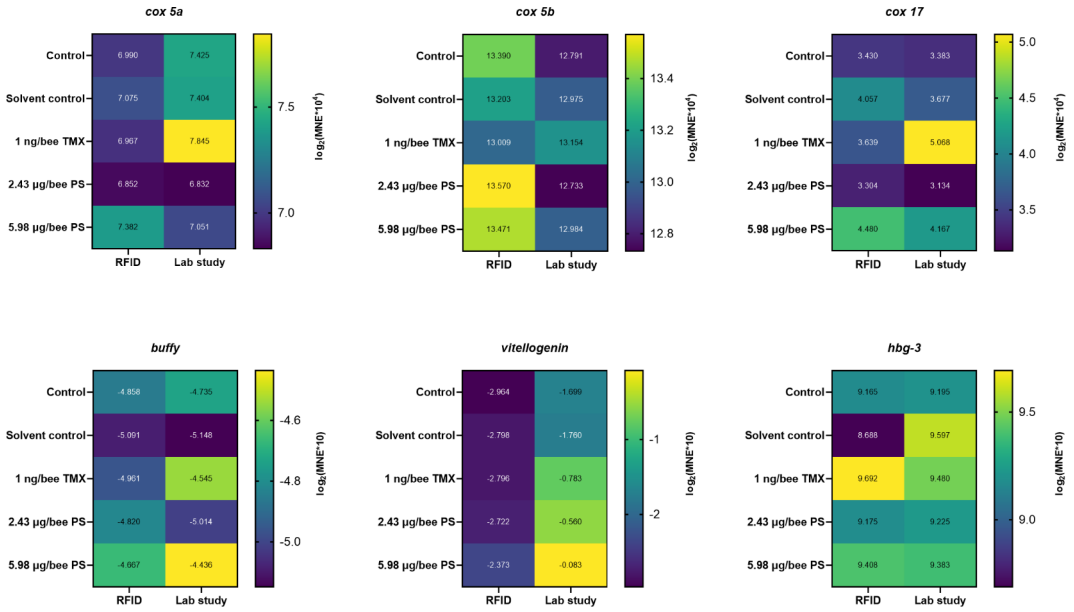


**Fig. 4**. Heatmaps of *cox5a*, *cox5b* and *cox17* (upper lane) and *buffy*, *vitellogenin* and *hbg-3* (lower lane) transcripts. Abundance of *cox*- and endocrine-transcripts after the RFID experiment and laboratory-based study without flight phase. The numbers represent the mean expression level of the transcripts. The scale on the right side indicates a color code for the log2 transformed mean normalized expression of the transcript for better visualization.

short-term hyperactivity after acute thiamethoxam exposure. Honey bees were able to fly faster and longer than the control group after exposure to 1.34 ng/bee thiamethoxam in the flight mill, yet after a chronic exposure (i.e. 1–2 days of exposure) their flight distance, duration and speed were significantly reduced. Subsequently, chronic exposure resulted in an overall reduced flight activity[42]. A similar flight behavior was also observed in bumble bees after exposure to an acute dose of 10 ppb imidacloprid[43]. In their flight mill experiment, the exposed bees initially showed a higher velocity but ended their flight earlier when compared to controls. This could be due to increased energy expenditure, leading to faster muscle fatigue[43].

Interestingly, flight duration varied across colonies suggesting that distinct genetic characteristics of the foragers can affect foraging behavior[44,45]. Other studies had also observed different responses among colonies to the same exposure scenario in terms of flight activity[38], demonstrating the relevance of accounting for colony-level genetics when interpreting ecotoxicological data[46]. It is known that the genetic variations amongst colonies can result in variable detoxification capacities, leading to differences in insecticide sensitivity and susceptibility[47].

The molecular gene expression analysis of returning bees within two hours revealed no significant transcriptional alterations when compared to the controls. In contrast, a previous study demonstrated a correlation between prolonged home flight duration and increased *cox5a* transcript after thiamethoxam exposure[29]. However, irrespective of the treatment group, our data revealed a significant correlation between prolonged homing flight duration and increased expression levels of *cox5a*, *cox5b*, *cox17* and *hbg-3*, suggesting that flight activity has an impact on gene regulation. As flight requires a high energy demand which is met by an increased density of mitochondria in the flight muscles, generating elevated adenosine triphosphate (ATP) by means of oxidative phosphorylation[48], this could explain the increased *cox5a* and *cox5b* expression level in the brain of analyzed honey bees. Not only flying, but also the detoxification of xenobiotics requires energy resources[49]. The high energy demands of detoxification have the effect of increasing the regulation of proteins involved in ATP synthesis[49], which is why this detoxification process could also be fatiguing, resulting in prolonged return time. This would also confirm our observation of reduced out-hive time after treatment compared to solvent control. To confirm this assumption of detoxification during flight activity, transcripts associated with metabolic enzymes could be additionally analyzed in a future RFID homing flight test.

Interestingly, the expression level of *hbg-3* scattered strongly in both the RFID test and laboratory-based study, despite the foragers being of the same age (i.e., ~ 20 days). The expression and enzymatic activity of *hbg-3* increases during maturing of honey bees between day 18 and 24[50] and is thus expressed at its highest in foragers and to a far less extent in nurse bees[25]. Despite the age-dependent regulated role of honey bees, the transition from nurse bees to foragers is also influenced by the social environmental factors including number of nurse bees and foragers, foraging rate, number of larvae; and can be accelerated, delayed, or even reversed depending on the needs of the colony[51]. Therefore, the developmental state of bees of the same age can vary, which may explain the large variance in *hbg-3* levels. The observed relation between prolonged homing flight duration and increased *hbg-3* level is an interesting discovery. One possible scenario could be that precocious bees with more flight experience, and thus better trained flight muscles, may have had sufficient energy to collect pollen after exposure to thiamethoxam and pyraclostrobin. Alternatively, yet not mutually exclusive, precocious bees at the

time of the experiment may have already been weaker than the others due to having already begun foraging earlier. Subsequently, they may have performed more flights, and therefore needed longer to return to their hive. Past studies on precocial foragers showed that they indeed completed fewer foraging flights and the risk of dying in their first flights is significantly higher. As the younger foragers are inefficient at collecting pollen and nectar, the transition begins earlier and foraging becomes more inefficient over time, which may ultimately lead to the colonies no longer able to meet food requirements[52].

In contrast to the RFID test, the exposure of thiamethoxam and pyraclostrobin of pollen foragers in the laboratory study without the subsequent homing flight phase had a significant influence on energy metabolism. Exposure to 1 ng/bee thiamethoxam induced an upregulation of *cox17* and pyraclostrobin inhibited the expression of *cox5a* (2.43 µg/bee) and *cox6c* (2.43 µg/bee and 5.98 µg/bee) confirming the effects of the two test substances on the expression of transcripts linked to energy metabolism. Due to the large dispersion of the data and relatively small sample size, no more significant changes were found. Chronic exposure of honey bee foragers to 5.59 ng/bee thiacloprid also affected the expression of transcripts encoding enzymes involved in oxidative phosphorylation[28]. Likewise, exposure of foragers to three different fungicides (azoxystrobin, chlorothanolin and folpet) altered the expression of several *cox*-transcripts[53]. Pyraclostrobin altered the expression of transcripts of oxidative phosphorylation in 20 days old foragers of *Apis cerana* exposed for ten days to 0.183 mg/ml[54]. Moreover, pyraclostrobin inhibited the oxidative phosphorylation and affected the mitochondrial function of honey bee negatively[23]. Furthermore, the fact that we found a significant shift in the expression of cox transcript in the laboratory study, but not in the RFID test, could support the theory of short-term excitation resulting in depletion and would be consistent with the result from the correlation analysis that the exposed bees took longer to fly home. We assume that this short-term hyperactivity could lead to short-term response in gene expression that could be detected in a laboratory-based study, but not in homing flight experiment because we may have missed the time point (subsided gene expression activity). This could explain why we detected significant transcriptional alterations in the laboratory study but not in the RFID homing flight experiment. As with time outside the hive, it is not clear why some bees took longer to return. Plausible reasons may be fatigue due to impaired energy metabolism, disorientation, or simply due to increased foraging activity. Although the *cox*-transcripts showed different expression patterns in laboratory exposure and RFID experiment according to heatmap, they were affected by pesticide exposure and may potentially function as biomarkers indicative of modified flight behavior after exposure to PPPs. Nevertheless, it is pertinent to note that, as per the data presented in this study, the definitive identification of biomarkers remains elusive at this juncture. Unfortunately, the data presented here does not enable such conclusions as varying gene expression patterns were observed between the RFID experiment and the laboratory study. The different gene expression patterns are likely a result of the flight activity of the RFID bees compared to laboratory bees as the exposure phase was identical between the two groups. This is undermined by the above previously discussed positive correlation between gene regulation and flight duration. It is known that the flight activity of foragers has a strong influence on gene expression[55]. The approach used here was not suitable to identify potential biomarkers for altered flight activity due to pesticide exposure. However, this does not mean that such biomarkers do not exist, as only a limited number of transcripts were analyzed in this study.

In summary, this is the second bee study combining lethal (i.e. survival) as well as sublethal behavioral (i.e. homing flight ability and flight behavior) and molecular (i.e. gene expression) data. Additional data along the same lines is of high relevance as it will enable a more holistic understanding of the potential effects of PPPs on honey bees and potentially other non-target organisms. Current risk assessments have a strong focus on testing the effects of PPPs only on the endpoint measure mortality and to a far less extent on critical sublethal parameters, such as flight activity, molecular gene expression, or fitness. Further data combining these endpoint parameters are required and would enable an enhanced mechanistic understanding of how field relevant exposure scenarios of PPPs are affecting insects.

## Methods

### Chemicals and treatments

Thiamethoxam and pyraclostrobin (purities of all > 99%) were purchased from Sigma-Aldrich (Buchs, Switzerland). Stock solutions for each compound were prepared in 100% acetone and diluted into 40% sucrose-solution to the final exposure concentrations. A thiamethoxam exposure of 1 ng/bee was chosen which is a factor 5 lower than the LD50[58], yet is still known to show significant effects on worker homing abilities[29,61]. For pyraclostrobin (PS), a field-relevant concentration of 2.65 µg/bee[24] was applied. Since pyraclostrobin has a low toxicity to bees, with an oral $LD_{50}$ above 100 µg/bee[56], a dose of 100 µg/bee was included in the study. Pyraclostrobin has a low solubility in water, hence final acetone concentration in sucrose-solution was 5%[57] to enhance its solubility in the aqueous sucrose solution. Due to pyraclostrobin's poor solubility, analytic verification of pyraclostrobin solutions was conducted with high-performance liquid chromatography and mass spectrometry (HPLC MSMS). The analytical results indicate that the final concentration of pyraclostrobin in the aqueous sucrose solution of the two treatment groups was 2.43 µg/bee and 5.98 µg/bee, respectively. A summary of measured concentrations is shown in supplementary Table S1. For uniform procedure, final sucrose-solutions with thiamethoxam also contained 5% acetone. Five treatment groups were set up (40% sucrose control group (C), 5% acetone solvent control (SC), 1 ng TMX/bee, 2.43 µg PS/bee and 5.98 µg PS/bee) in three non-related, queenright *Apis mellifera* (L.) colonies where each colony represented an independent replicate. Each treatment group contained 40 individual forager bees, from which 20 were randomly selected for gene expression analysis and the remaining 20 were used for the longevity assessment. This resulted in a total of 200 bees per colony. Each pollen forager was single fed with either 40 µl of 40% sucrose solution (i.e., control group), or 40% sucrose solution containing 5% acetone (i.e., solvent control), or 40% sucrose solution containing 5% acetone and 1 ng/bee thiamethoxam ($LD_{50}$: 5 ng/bee[58]), 40% sucrose solution containing 5% acetone and 2.43 µg/bee (nominal

concentration: 2.65 µg/bee) or 5.98 µg/bee pyraclostrobin (nominal concentration: 100 µg/bee, (Table S2). The bees were kept individually in cages in an incubator under complete darkness until they entirely consumed the provided treatment dose.

## Orientation flight, RFID chip tagging and exposure phase

To obtain pollen foragers of a known age-cohort for the homing flight RFID experiment, brood combs with capped cells, expected to hatch within ~24 h, were removed from the respective experimental colonies and transferred to an incubator at 33 °C ± 2° C and 60% relative humidity. The following day, 2,500-3,000 newly emerged individuals were color coded on the thorax using a non-toxic, water-based permanent marker (Uni Posca, Mitsubishi Pencil Company Ltd., Japan). This initial marking was done to ensure we could identify workers on day 20 post-emergence, when workers were expected to become foragers[59]. After marking, the newly emerged bees were placed back to the corresponding donor (i.e., natal) colony. On day 20, marked workers were recollected at the hive entrance and covered with a non-toxic pink powder (Pigment Laser Red Fluorescent A3, T series, COLOREY SAS, France). This second coloring step was done for the orientation flight – a necessary step to ensure that the foragers used for the homing flight experiment were familiar with their surroundings and homing route. The powdered bees were then brought to the release location (one kilometer distance to their hive) and released. At the hive entrance, ≥200 returning powdered bees were collected, transferred to the laboratory, and again maintained under complete darkness with access to Apifonda® (Südzucker AG, Germany) prior to being RFID chip tagged. During the collection phase, bees were provided with Apifonda® to ensure they had sufficient carbohydrates and kept in complete darkness in an incubator. After the orientation flight, the foragers ($n = 200$ bees: five treatment groups with 40 bees per treatment group) were individually tagged with a RFID chip (MAJA 13.56 MHz RFID system, Microsensys GmbH, Germany) and randomly allocated to either the gene expression analyses ($n = 20$ bees) or used to measure longevity ($n = 20$). The chip was fixed on dorsal thorax of each bee with TempoSIL 2® dental cement (Coltène Holding AG, Switzerland). Immediately after tagging bees were kept in individual cages for the treatment exposure phase.

## RFID system

The RFID MAJA system (Microsensys GmbH, Germany) consists of a data carrier, called a tag or chip, and a reader. The reader emits weak electromagnetic waves that are reflected by the chip. This means that every bee that carries a RFID chip and passes the reader is registered. The information (i.e., identification of a skipped bee) is recorded without contact and stored in the reader's memory. Each chip has a unique identification code (UID). This code and the exact time of the event (date, hour, minute and second) are recorded electronically as a real-time record. Each hive was equipped with eight RFID readers in a two-row configuration at the entrance of the hive (four entrances of the hive with two readers each). With the two-row configuration, the direction of the bees (i.e., hive in or out) can be determined by the order in which the bees pass the paired readers per row. Based on this data set the total out-hive time per bee was assessed and used as a proxy to determine the approximate foraging time spent during an individual's lifespan. A picture of the RFID system installed at the entrance of a honey bee hive is shown in Fig. S3.

Sorting and evaluation of the flight data was performed with the R-based software packages version 4.1.2, readxl version 1.4.2, rlang version 1.1.1, lubridate version 1.9.2 and tidyverse version 2.0.0. In an initial step to calculate the return rate [%], the first registered measurement from any sensor was used for each UID (i.e., bees passing the readers with RFID chip). According to the affiliation of a UID to a colony, the difference between release time and first measurement was calculated for each bee (return rate and return time after treatment and release). Measurements exceeding 24 h after release were filtered for further calculations (n (C): 55; n (SC): 51; n (1 ng/bee TMX): 39; n (2.43 µg/bee PS): 49 and n (5.98 µg/bee PS): 47, Table S4) (i.e., time spent outside of hive (out time [h]) and longevity [d]). In a second step, the flight behavior of the remaining bees was observed over a period of up to 40 days (out time). Our data was further filtered according to the follow predefined conditions: An entry into the hive or an exit from the hive was only registered as valid, if a bee passed both readers within a 10 s period. Furthermore, to avoid false positive results for bee activity during the night where bees during warm evenings will stay in front of the hive, all measurements recorded before sunrise and after sunset were removed from the dataset. In addition, out-hive times under two minutes were excluded and not considered as a foraging trip. Lastly, for each bee and each day, we checked whether the first measurement after sunrise was an outgoing measurement or not. Subsequent measurements are then further validated as to whether there is a valid sequence of incoming and outgoing foraging trips. Bees performing invalid flights (see Table S4) were registered during the experimental period and were removed from the data. Finally, with the remaining data set (see Table S4), the out time (i.e. flight time of each foraging trip) was calculated for each bee and each combination of outgoing and incoming measurements. The generated data set was used as the basis for further calculations of flight behavior during the lifespan of each marked and treated bee.

## RFID homing flight

After the exposure phase, individuals were transferred to the same release location as for the orientation flight and released for the homing flight experiment. Bees that returned within two hours were recaptured at the hive entrance and directly stored in dry ice for subsequent gene expression analysis (n (C): 36; n (SC): 29; n (1 ng/bee TMX): 34; n (2.43 µg/bee PS): 22 and n (5.98 µg/bee PS): 18, Table S3). To register the arrival time (i.e., homing flight duration [s]) of the foragers used for gene expression analysis, the pre-installed sensor at the entrance was triggered manually. Returning bees (> 2 h time for homing flight)that were not sampled for the gene expression analysis (n (C): 9; n (SC): 9; n (1 ng/bee TMX): 4; n (2.43 µg/bee PS): 14 and n (5.98 µg/bee PS): 17, Table S3) (i.e., bees having homing flight durations beyond two hours) were automatically recorded by the RFID readers at the hive entrance to monitor returning rate [%] upon initial release and included to longevity assessment

(i.e., survival [d]), and total time spent outside the hive during their lifespan (i.e., out time [h]). Bees that were released to measure homing flight duration that did not return within 24 h were considered as non-returned bees. In total the experiment was repeated three times using foragers of three different colonies on three different days (1.06., 10.06. and 27.06.2021). A graphical overview of the whole experimental procedure is shown in Fig. S1. The weather conditions on each of the three days were similar (maximum temperature: 24.3–26.8 °C, temperature at noon: 21.8–23.9 °C, no rain for all three days, sunlight: 9.4–14.4 h; Fig. S2). Flight behavior and homing abilities were analyzed according to the OECD 332[60] and Jeker & Grossar (2020)[61].

## Laboratory study

To investigate whether transcriptional alterations are found after PPP exposure without completing a homing flight, the following approach was carried out: Pollen foragers of the same age-cohort as used in the homing flight experiment were collected and exposed to thiamethoxam or pyraclostrobin using the identical procedures as described for the field test. After the exposure phase, individuals were however directly stored on dry ice for the molecular analysis.

## Gene expression analyses

RNA isolation, reverse transcription, and qPCR: The entire brain of the frozen bee was collected by opening the cranium with a scalpel and forceps. Total RNA of each brain was isolated according to the manufacturer's instructions (RNeasy mini kit, Qiagen, Switzerland). The quantification of total RNA was measured by NanoDrop spectrophotometer (Witec AG, Switzerland). 500 ng of total RNA were reverse transcribed using M-MLV Reverse transcriptase 200 U/µl (Promega, USA) and 1 µl random hexamer primer (Roche, Switzerland). First strand cDNA was used as template to perform two step qPCR using SYBR-Green Fluorescence (FastStart Universal SYBR Green Master, Roche Diagnostics, Switzerland) and the primer pairs listed in Table S5. The efficiency of each primer pair was assessed as described in Christen, 2023[25]. Real time PCR amplification was performed on Bio-Rad CFX96 RealTime PCR Detection System (Bio-Rad Laboratories, Inc., USA) under the following conditions: 95 °C for 10 min as initial denaturation, followed by 40 cycles at 95 °C for 15 s and 59 °C for 60 s. Melting-curve analyses were performed after each run to ensure the formation of specific products. The reference gene *ribosomal protein S5* (*rpS5*) was used to normalize gene expression. This selection was based on the stable transcription of *rpS5* across seasons[62] and has been used in previous studies[29]. *RpS5* was stably expressed across all treatment groups (Fig. S4). The selection of transcripts analyzed was based on previous studies showing altered expression of *cox*-transcripts *(cox5a*, *cox5b*, *cox17)* and endocrine transcripts (*vitellogenin*, *buffy*, *hbg-3*) after insecticide and fungicide exposure[26,53]. Moreover, a correlation between *cox*-transcript expression and homing flight duration has been revealed[29]. These *cox*-transcripts are of particular interest because they are subunits of cytochrome c oxidase (complex IV) which is an essential enzyme in the mitochondrial electron transport chain that drives oxidative phosphorylation. Therefore, the inhibition of *cox*-transcripts likely leads to a reduction in ATP synthesis[23], potentially reducing flight abilities due to an energy insufficiency[63]. In contrast, the tested endocrine transcripts are on the one hand related to the physiological transition of inhive bees (e.g., nurses) to foragers and on the other hand they are known to affect foraging behavior as well as homing success[12]. The transition from inhive bees to foragers is regulated by *vitellogenin*[64], whereas the *hbg-3* gene encodes the α-glucosidase lll which is primarily expressed in active forager bees. This enzyme converts sucrose in nectar to glucose and fructose[65]. Lastly, *buffy* is a hypopharyngeal gland (HPG)-specific gene and is commonly expressed at high levels in inhive bees[65].

## Processing of qPCR data

To obtain the mean normalized expression level of the transcripts of interest, the raw qPCR data was processed according to the equation described by Simon Perikles[66]:

$$\mathrm{MNE} = \frac{\left(\mathrm{E}_{\mathrm{reference}}\right)^{\mathrm{CTreference,mean}}}{\left(\mathrm{E}_{\mathrm{target}}\right)^{\mathrm{CT\,target,mean}}}$$

MNE stands for mean normalized expression, which is used for qPCR data and con-siders the different efficiencies of PCR amplification for the target ($E_{target}$) and the ref-erence ($E_{reference}$); $E_{ref}$ is reference gene efficiency; $E_{target}$ is target gene efficiency; $Ct_{ref;\ mean}$ is mean Ct value for reference gene; and $Ct_{target;\ mean}$ stands for mean cycle threshold (Ct) value for target gene. The mean normalized expression level of exposed pollen foragers was always compared with the mean normalized expression level of solvent group (40% sucrose solutions with 5% acetone) to determine the effects, since 5% acetone were added to thiamethoxam and pyraclostrobin for better dissolution.

## Data preparation and analyses

Statistical analyses and figures were performed using both GraphPad Prism 9 (version 9.1.0) as well as STATA17[67]. Outcome variables (i.e., homing flight duration [s], return rate [%], out time [h], and gene expression [MNE]) were tested for normality by using the Shapiro-Wilk's test and homogeneity of variances with the Levene's test and the appropriate statistical methods were chosen accordingly. In case of normal distribution, one-way analysis of variance (ANOVA), respectively two-way ANOVA for return rate, followed by Sidak's test for multiple comparisons was used to compare treatment means with solvent control. If data normality was not met, the non-parametric Kruskal-Wallis test was applied using the Dunn's test to account for multiple comparisons among treatment groups. The survival analysis was performed using the STATA function *mestreg* for multilevel survival models, wherein treatment was set as the fixed explanatory variable and individual bee age at exposure as well as colony ID were incorporated as covariates. A survival curve (Kaplan Meier plot) was used to visually display the

survival data. Further, generalized linear mixed-effects models were fit to assess potential relationship between homing flight duration, out time [h] and gene expression using the STATA function *meglm*. Individual forager bees were considered as independent whereas exposure, individual genes, and homing flight duration and out time [h] were included as explanatory variables and wherever necessary run (i.e., colony) and/or age of exposure were added to the model as a co-variate. As homing flight duration, out time [h] and gene expression data were not normally distributed (Shapiro-Wilk's test, $p < 0.001$) they were all fitted to a negative binomial model using the *menbreg* function. Generalized logistic mixed models were applied to test for treatment differences for the binary outcome variable return rate [%] using the function *melogit*. Here, the conditional distribution of the regression given the random effect (i.e., run) was Bernoulli. Before running the full models, a test for multi-collinearity was run using the variance inflation factor as our metric and the function *vif*. Further, both a likelihood ratio (LR) tests as well as the Akaike information criterion (AIC) were used to test for models of best fit using the functions *lrtest* and *estat ic*, respectively. XY scatter plots were used to visualize the results for the genes that revealed significant differences between treatment groups. The qPCR results as well as survival and homing flight duration data are given as corrected means ± standard error of means (SE).

## Data availability

The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

Received: 17 April 2024; Accepted: 16 September 2024
Published online: 01 October 2024

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## Acknowledgements
We would like to thank Dr. Christina Kast from the Swiss Bee Research Center for her efforts in conducting the chemical analysis of treatment solutions.

## Author contributions
AK, LJ, DG and VC performed the experimentsAK, LJ, DG, VC and LS wrote the main manuscript textJM mainly analyzed the raw data of the RFID-experimentLS mainly performed most of the statistical analysisAll authors reviewed the manuscript.

## Declarations

## Competing interests
The authors declare no competing interests.

## Additional information
**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-73235-9.

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