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Insecticide exposure alters flight-dependent gene-expression in honey bees, *Apis mellifera*

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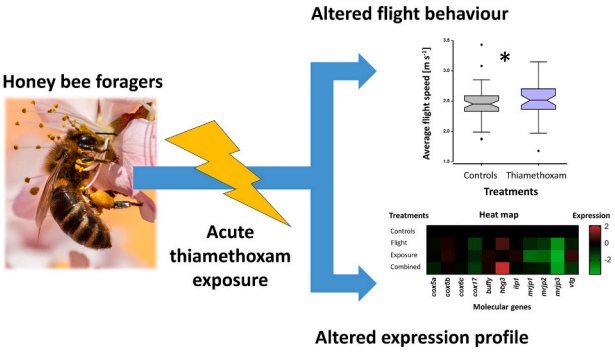
HIGHLIGHTS

- Acute thiamethoxam exposure resulted in hyperactive flight behavior in foragers.
- Thiamethoxam impairs transcription profiles related to ATP synthesis.
- Physical exertion (i.e., flight) exacerbates the effects of exposure at the molecular level.

GRAPHICAL ABSTRACT



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ABSTRACT

The increased reports of wild bee declines and annual losses of managed bees pose a significant threat to biodiversity and agricultural productivity. While these losses and declines are likely driven by various factors, the exposure of bees to agrochemicals has raised significant concern due to their ubiquitous use and potential adverse effects. Despite numerous studies suggesting neonicotinoids can negatively affect bees at the behavioral and molecular level, data linking these two factors remains sparse. Here we provide data on the impact of an acute dose of the neonicotinoid thiamethoxam on the flight performance and molecular transcription profiles of foraging honey bees (*Apis mellifera*). Using a controlled experimental design with tethered flight mills, we measured flight distance, duration, and speed, alongside the expression of genes involved in energy metabolism, hormone regulation, and biosynthesis. Acute thiamethoxam exposure resulted in hyperactive flight behavior but led to significant dysregulation of genes associated with oxidative phosphorylation, indicating potential disruptions in cellular energy production. These molecular changes were particularly evident when bees engaged in flight activities, suggesting that the combined stress of pesticide exposure and physical exertion exacerbates

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negative outcomes. Our study provides new insights into the molecular mechanisms underlying neonicotinoid-induced impairments in bee physiology that can help understand the potential long-term consequences of xenobiotic exposure on the foraging abilities of bees and ultimately fitness.

1. Introduction

Considerable attention has been devoted to understanding the potential contribution of neonicotinoid insecticides to the widespread declines of wild bees, and increased annual losses of managed honey bee colonies in the Northern Hemisphere (e.g., Sánchez-Bayo, 2011; Godfray et al., 2014; Zattara and Aizen, 2021; Gray et al., 2022). These insecticides are frequently used in agricultural and horticultural settings as well as private gardens to control a broad spectrum of pests (Jeschke and Nauen, 2008). Due to their systemic nature, neonicotinoids are frequently found contaminating nectar and pollen, as well as plant fluids (Goulson, 2013), thereby becoming a potential threat to non-target organisms, such as pollinating insects (Simon-Delso et al., 2015; Main et al., 2018). Indeed, numerous studies have shown that field-relevant concentrations can have lethal and sub-lethal effects on bees, at the physiological, behavioral, and molecular level (Pereira et al., 2020). While a ban on the outdoor usage of specific neonicotinoid insecticides (i.e., thiamethoxam, clothianidin and imidacloprid) has been implemented in the European Union since 2013, they are still amongst the most extensively used insecticides globally (Klingelhöfer et al., 2022).

Understanding the impact of neonicotinoids on honey bee flight performance (e.g., distance, duration, or speed) is of particular interest as it governs their capacity to exploit resources within their habitat, thereby determining colony dynamics, growth and fitness (Raine and Chittka, 2008; Moritz and Southwick, 2012; Becher et al., 2014). Subsequently, several studies have shown that exposure to neonicotinoids can alter flight patterns (Fischer et al., 2014), decrease flight duration (Tosi et al., 2017) and impair homing ability (Tison et al., 2016). Such affects are argued to compromise foraging efficiency in bees and lead to downstream negative effects on fitness (Gill et al., 2012; Stuligross and Williams, 2020). Current data suggest that exposure can induce changes in honey bee gene and transcript expression profiles, particularly in genes associated with oxidative phosphorylation and hormone regulation (Christen et al., 2016; Fent et al., 2020; Fischer et al., 2024). Oxidative phosphorylation is a fundamental metabolic process that generates adenosine triphosphate (ATP) – the primary energy source of cells (Senior, 1988). Any disruption in oxidative phosphorylation pathways will inevitably compromise cellular energy production and lead to increased oxidative stress, which can cause damage at the cellular, tissue and organismal level (Xu et al., 2022). Furthermore, neonicotinoids have been documented to disrupt biosynthesis, endocrine and hormone pathways (Christen et al., 2018; Erban et al., 2019; Fent et al., 2020; Christen et al., 2021), which are essential for regulating development, metamorphosis, and reproduction in insects (Bellés et al., 2005). Yet, despite numerous studies investigating the impact of neonicotinoid exposure on bees, a gap remains in our understanding of the molecular mechanisms that may underlie altered behavior and/or impaired physiology.

Reasons for the lack of such data are likely due to the inherent difficulty of conducting such studies. For instance, assessing flight performance of individual bees under field conditions - besides often being labor and cost intensive - poses a significant challenge due to uncontrollable confounding factors (e.g., weather conditions or drifting of bees (Nunes-Silva et al., 2020)). While laboratory studies may differ from observations under natural conditions (Thompson and Maus, 2007; Godfray et al., 2014), controlled laboratory studies are crucial for obtaining ecotoxicological data that may elucidate toxicological mechanisms. Tethered flight mills are an ideal experimental tool to assess sublethal effects such as insect flight capacity and behavior, by enabling flight to be recorded under controlled settings (e.g., Jones et al., 2015, 2016; Dällenbach et al., 2018; Minter et al., 2018). While past studies have used this method to measure the influence of neonicotinoids on flight performance of insects (e.g., Blanken et al., 2015; Tosi and Nieh, 2017; Tosi et al., 2017; Kenna et al., 2019; Cibotti et al., 2024), none have implemented this tool to unravel the potential link between molecular and behavioral responses post exposure.

Tethered flight mills offer an elegant method to explore the possible link between sublethal effects of an acute field-relevant dosage (i.e., 1 ng bee^{-1}) of the neonicotinoid thiamethoxam on flight performance and molecular transcription expression on pollen foraging honey bees, *Apis mellifera*. By doing so, we sought to identify molecular markers that may be used to predict potential effects of neonicotinoid exposure on honey bee physiology. To address this, we conducted a fully crossed experimental design to investigate the potential interactive effects of neonicotinoid exposure and flight performance on a subset of target transcripts associated with energy metabolism (i.e., cytochrome *c* oxidase (*cox*) genes), endocrine and hormone regulation (e.g., vitellogenin (*vg*), insulin-like peptides (*ilps*)), and biosynthesis (i.e., major royal jelly proteins (*mrjps*)). Considering previous studies (e.g., Tosi et al., 2017; Wu et al., 2017; Christen et al., 2021), we hypothesize that thiamethoxam exposure will alter both flight performance and transcription profiles, thus enabling the identification of potential correlations between physiology and molecular markers.

2. Methods

The experiment was conducted between September 2020 and March 2021 at the Institute of Bee Health, University of Bern, Swiss Bee Research Centre, Agroscope, Switzerland and the University of Applied Sciences and Arts Northwestern Switzerland, using three local, non-related and queenright *Apis mellifera* colonies.

2.1. Thiamethoxam solution

Pesticide and control solutions were prepared following standard protocols (OECD, 1998; EFSA, 2013). In brief, pure analytical grade thiamethoxam (Sigma-Aldrich 37924-100MG-R, UK) was dissolved in double-distilled water to produce a primary stock solution (1 mg ml^{-1}). Acetone was added as a solvent, accounting for <0.1 % of the volume in the stock solution. Likewise, a control stock solution was produced containing the identical volume of acetone to account for potential inadvertent effects of the solvent. The stock solutions were refrigerated at 4 °C in a tin-foil-covered flask to ensure complete darkness, thereby preventing potential UV-degradation of the active ingredient. The thiamethoxam solution fed to the bees was prepared daily by diluting the stock with 20 % [*w/v*] sucrose solution to a final exposure concentration of 1 ng thiamethoxam bee^{-1}. In our study, we used honey bee foragers and thus the thiamethoxam levels detected in nectar provide the most relevant exposure levels. Thiamethoxam contamination levels in nectar of plants and crops can vary widely across space and time (Pisa et al., 2015), ranging anywhere between <1 to beyond 75 ng g^{-1} (Bonmatin et al., 2015; Calvo-Agudo et al., 2019; Zioga et al., 2020; Zhang et al., 2023). Therefore, the exposure level applied in our study (1 ng g^{-1}) is environmentally realistic and reflects levels that have been applied in previous studies (Tosi et al., 2017; Tosi and Nieh, 2017; Siviter et al., 2021a). To confirm the pesticide preparation was adequately performed, high-performance liquid chromatography (HPLC; Agilent 1290 Infinity II) coupled with mass spectrometry (MS/MS; Aligent 6495C tandem quadrupole) was conducted following Schaad et al. (2023). The desired concentration was confirmed by

testing two random samples from both the thiamethoxam (i.e., 0.93 and 1.09 ng g^{-1}) and control feeding solutions (i.e., below limit of detection (LOD) 0.4 ng g^{-1}).

2.2. Exposure of forager bees

The acute exposure scenario was conducted following a modified version of Christen et al. (2021). In brief, 30 returning forager bees with pollen filled corbiculae were caught at the entrance of three local honey bee hives and placed in a standardized hoarding cage [100 cm^3] (Williams et al., 2013). Once the 30 foragers were sampled, they were immediately transferred to the laboratory and kept in an incubator at 30 °C and 60 % relative humidity (RH). To enhance the likelihood of bees consuming their later exposure treatment, individuals were subjected to a one-hour starvation period. The starvation period would also ensure that the experimental bees would consume nectar that they may have collected while foraging and stored within their honey crop. Thereafter, bees were carefully removed from the hoarding cage using forceps and the body mass of each specimen was recorded to the nearest 0.1 mg using an analytic scale (Mettler Toledo AT400). Bees were then placed in an individual feeding cage and randomly allocated to either the thiamethoxam or control treatment groups, where they received either 20 μL of 30 % [*w/v*] sucrose solution (control), or 20 μL of 30 % [w/v] sucrose solution containing 1.0 ng thiamethoxam (treatment). Following Kenna et al. (2019), bees that consumed the first 20 μL of solution within 10 min were then provided an additional 20 μL of 30 % [w/v] sucrose solution which needed to be consumed within an additional 10-minute period. Bees that successfully consumed 40 μL of sucrose solution within 20 min were then placed back into the incubator (30 °C and 60 %) for 20 min to enable the bees to metabolize the sucrose solution. According to estimates, a feeding volume of 40 μL of sucrose is sufficient for a bee to forage for roughly 1 h (Rodney and Kramer, 2020). Bees that did not consume the first or second feeding dosage were excluded from the experiment, whereas those that did were then either used for the tethered flight mill assessment or frozen at −24 °C for later analyses of gene expression. For each round of 30 bees collected at the hive entrances, a maximum of eight bees (i.e., four per treatment) were assessed on the tethered flight mills and the remaining bees were used as 'baseline' individuals for the molecular assessment. To obtain a sufficient sample size ($N > 30$ bees per treatment), the experiment was repeated 14 times over the course of three consecutive days in early September. In total, 98 individuals ($N_{control} = 53$; $N_{insecticide} = 45$) were used for the flight mill experiment, whereby the three hives were evenly represented ($N_{hive\ 1} = 32$; $N_{hive\ 2} = 31$; $N_{hive\ 3} = 35$) and evenly distributed across both treatments.

2.3. Tethered flight mills

To investigate the flight behavior of forager honey bees, we used computerized tethered flight mills as described in Jones et al. (2016). In brief, the flight mills were designed at Rothamsted Research (patent: Lim et al., 2013) and consist of a lightweight wire arm suspended between two magnets, which results in negligible resistance against the turning of the arm. Thus, even weak fliers can turn the mill and rotate in a horizontal plane. A pin was carefully glued to the thorax of a honey bee and attached to one end of the mill arm, using a contact adhesive. A striped black and white disc attached to the axis turns with the arm. A light sensor detects the movement of the disc and records the distance flown [m], time spent flying [s] and flight speed [m s^{-1}]. The system used had eight mills, allowing eight bees to be flown simultaneously. Data for each individual was processed using a custom-written script (by K.S.L.) in Matlab (Jones et al., 2015). Further details on the design of the tethered flight mills can be found in Jones et al. (2016).

Flight trials were conducted throughout the day during 09:00–16.00. Four individuals per treatment were evenly allocated for each round of eight flight mill assessments. All experiments were performed under regulated climatic conditions (26 °C and 55 % RH) with indirect light from a nearby window. Bees with obvious signs of damage to the wings were excluded, whereas bees that still had their corbiculae filled with pollen were included and the presence of pollen was accounted for in the statistical analyses. All individuals ($N = 98$) were randomly assigned to one of the eight flight mills and were flown in a clockwise rotation. Immediately after placing the bees on the flight mills, they were provided with a styrofoam ball which was used as a platform for the bees to rest. To initiate flight, the styrofoam ball was removed as simultaneously as possible from all bees. Bees that did not start flying after the Styrofoam ball was removed were stimulated to fly by putting a finger under their body and then removing it. If the bee still did not fly, it was provided with the Styrofoam ball again and given 5 min to rest before removing the ball. If they still did not fly, they were considered as 'non-flyers'. All bees that flew were left on the flight mill until they stopped flying. To ensure that bees were exhausted and could no longer fly, individuals were stimulated to fly as previously described over a period of 3 min. When the bees finished their flights, they were removed from the mill, placed in an Eppendorf tube and stored at −24 °C.

2.4. RNA isolation, reverse transcription and qPCR

The brain of frozen bees was removed in total by opening the head using a scalpel and forceps. Brain tissue was chosen due to the increased mitochondrial density, which is comparable to the flight muscle tissues, a necessary adaptation that caters to the substantial energy requirements proportional to neural signalling and the rigors of flight activity (Sargent et al., 2021). Furthermore, brain tissue is ideal because neonicotinoids specifically interact with the nicotinic acetylcholine receptors (nAChRs), which notably constitute the predominant excitatory neurotransmitter system within the central nervous system of insects (Buckingham et al., 1997). Total RNA from one brain was isolated using an RNeasy®Mini Kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. 500 ng RNA were reverse transcribed, and qPCR based on SYBR green fluorescence (SYBR green PCR master mix; Roche, Rotkreuz, Switzerland) was performed as described in Christen (2023). Primer sequences used in this study and qPCR efficiencies are given in SI Table 1. *ribosomal protein S5* (*rpS5*) was used as a reference gene for normalization for all gene expression analyses. This selection is based on the stable transcription of *rpS5* across seasons in previous studies (Jeon et al., 2020; Christen et al., 2021). To untangle potential alterations of mRNA abundance in the brain due to thiamethoxam exposure as well as flight activity, we included individuals in our analysis that were exposed to their respective treatments but did not fly (i.e., baseline controls and baseline thiamethoxam exposed) and individuals that were exposed to their treatments and successfully flew. Thus, the experiment had the following four treatment groups: Controls (no thiamethoxam, no flight activity), Exposed (thiamethoxam exposure; no flight), Flight (no thiamethoxam, active flight), and Combined (thiamethoxam exposed; active flight).

2.5. Statistical analyses

Statistical analyses were performed using STATA16 (StataCorp., 2019), and figures were created using NCSS 20 (Hintze, 2020) and Graph Pad Prism. All response variables were tested for normality using the Shapiro-Wilk's test and homogeneity of variances with the Levene's test. Subsequent statistical methods were then chosen accordingly. To determine differences between the control and thiamethoxam treatment, multilevel generalized logistic or linear (regression) models (GLMMs) were fitted using the functions *logit* or *meglm*. Individual bees were considered as the independent units, and exposure and flight activity were included as the explanatory fixed terms. Whenever necessary, each model included co-variates (i.e., body mass, corbiculae filled with pollen) as well as random effects (i.e., tethered flight mill ID or date and time of flight mill assessment). A stepwise backward elimination

approach was applied to determine the model of best fit (Wiegand, 2010). Best fit models were chosen by comparing every multi-level model to its single-level model counterpart using a likelihood ratio (LR) test and comparing different models with the Akaike information criterion (AIC) using the functions *lrtest* and *estat ic*, respectively. To assess potential relationships amongst explanatory variables (e.g., body mass or mean normalized expression (MNE)) and the dependent variables (e.g., total flight distance or average speed), generalized linear regression models (GLMs) was applied using the function *glm*, where individual bees were considered independent units and not grouped as in the previous models mentioned above. Whenever appropriate, the means ± standard error (SE) or medians ±95 % confidence intervals (CI) are given in the text. Median differences and their 95 % CI were calculated using the STATA16 package *somersd*. The function *cendif* calculates confidence intervals for Hodges–Lehmann median differences (or other percentile differences) between two groups.

A logistic model was applied to assess the successful flight rate [%] based on a Bernoulli distribution, where the fixed factor was 'treatment', and the co-variates were 'mass' and 'corbiculae filled'. 'Flight mill ID' was included as a random effect. All non-successful flyers ($N = 6$) were excluded from the following statistical analyses. The variables total flight distance [m], total flight duration [min], and average and maximum flight speed [m s^{-1}] were modelled using GLMMs with either a Gaussian, Gamma or Poisson distribution, respectively, whereby 'treatment' was the single fixed factor and co-variates (e.g., mass and/or corbiculae filled) as well as including flight mill ID as a random effect.

For the gene expression analyses, we used a random subsample ($N = 60$) of individuals from the control and thiamethoxam treatment that were placed on the flight mill (i.e., flyers), as well as both control and thiamethoxam individuals that were stored at −24 °C without having been placed on the flight mill (i.e., 'baseline' individuals). This enabled us to investigate the effect of thiamethoxam exposure and flight both individually, and in combination, on gene expression. Both treatments (i.e., exposure and flight) and their interaction term (cross-product term, '*exposure#flight*') were included in the models as fixed (explanatory) factors. If a model revealed a significant effect for either exposure or flight, it was followed up by a Bonferroni multiple pairwise comparisons test (*bmtc*) using the function *mcompare*(*bonferroni*). Honey bee mass and pollen presence were included as covariates in the models whenever necessary. Furthermore, correlations between gene expression and flight variables (e.g., total flight distance, or maximum speed) were explored using linear regression models fitted using the function *glm*. Only individuals from the flight treatment group were used for this analysis.

2.6. Interactions

If the interaction term in the GLMM was non-significant, we assumed the interaction to be neutral. However, if the interaction term revealed significance ($p < 0.05$), we calculated the interaction effect size following Siviter et al. (2021b). Details regarding the precise calculations can be found in the supplementary information (SI).

3. Results

3.1. Tethered flight mill assessment

Thiamethoxam exposure led to a higher flight success rate compared to controls (*melogit*; $z = 2.22$, $P = 0.027$), where flight success rates were 97.8 % and 90.5 % for exposed and controls, respectively. Irrespective of the treatment group, body mass had a negative effect on flight success ($z = -2.62$, $P < 0.01$). Flight success was not influenced by the presence of pollen in the corbiculae ($z = -1.26$, $P = 0.20$). Thiamethoxam exposure led to prolonged flight duration (*meglm*; $z = 2.01$, $P = 0.045$; Fig. 1A), resulting in exposed bees showing 24 % increased median flight duration (median 46 min, 95 % CI 39, 51) compared to controls (median 37.3 min, 95 % CI 30, 42). Total flight distance was longer for bees in the exposed treatment (*meglm*; $z = 2.10$, $P = 0.036$; Fig. 1B), whereby median total flight distance was 5.7 km (95 % CI 4.4, 6.7 km) and 7.1 km

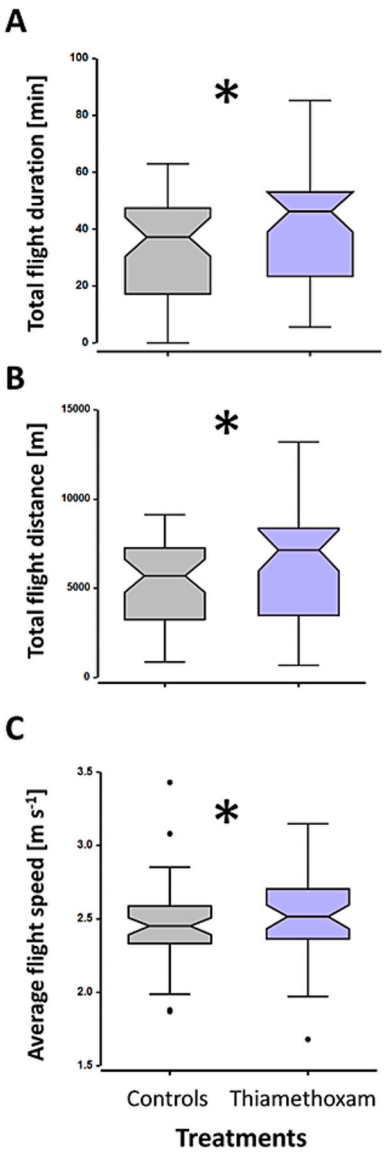

Fig. 1. Tethered flight mill assessment of female (worker) honey bees, *Apis mellifera*, exposed to the neonicotinoid insecticide thiamethoxam. Exposure revealed a significant positive effect on (A) total flight duration, (B) total flight distance and (C) average flight speed (all *P*'s < 0.05). The boxplots show the inter-quartile range (box), the median (black line within box), data range (horizontal black lines from box), and outliers (black dots). A significant difference between treatment groups is indicated by $*P < 0.05$.

Table 1
Summary of the linear mixed model output for the effects of neonicotinoid (thiamethoxam) exposure and flight, individually and in combination, on gene expression in female (worker) honey bees (*Apis mellifera*). Bold font text reflects significant statistical differences (i.e., $P < 0.05$) and up or down regulation of genes.

Category	Gene	Sample size	Residual df	Log likelihood	AIC	Explanatory variables	Coef.	Std. Err.	*z*-Value	*p*-Value	Lower	Upper	Gene regulation
Oxidative phosphorylation	*cox5a*	46	42	−240.79	10.64	Exposure	−0.24	0.12	−1.99	**0.05**	−0.47	0.00	**Down**
						Flight	−0.48	0.17	−2.77	**0.01**	−0.82	−0.14	**Down**
						Combined	−0.69	0.13	−5.06	<0.001	−0.95	−0.42	**Down**
	cox5b	46	41	−414.19	18.25	Exposure	1200.73	885.90	1.36	0.17	−535.56	2937.14	None
						Flight	−2739.16	1251.40	−2.19	**0.03**	−5191.95	−286.37	**Down**
						Combined	−2077.63	972.70	−2.14	**0.03**	3984.14	−171.13	**Down**
	cox6c	46	41	51.51	−2.02	Exposure	−2.17	175.00	−2.14	0.21	−2.61	1.25	None
						Flight	1.41	2.61	0.54	0.59	−3.69	6.52	None
						Combined	1.33	2.17	0.61	0.54	−3.93	5.59	None
	cox17	46	41	−169.20	7.57	Exposure	−0.57	0.12	−4.59	**<0.001**	−0.81	−0.32	**Down**
						Flight	−1.21	0.21	−5.74	**<0.001**	−1.63	−0.79	**Down**
						Combined	−0.78	0.15	−5.29	**<0.001**	−1.07	−0.49	**Down**
Endocrine regulation	*vg*	46	42	−46.39	2.19	Exposure	0.36	0.49	0.74	0.46	−0.61	1.33	None
						Flight	−0.28	0.60	−0.47	0.64	−1.46	0.89	None
						Combined	−0.52	0.64	−0.81	0.42	−1.77	0.74	None
	ilp-1	46	40	−73.17	3.39	Exposure	−0.06	0.07	−0.89	0.37	−0.21	0.07	None
						Flight	−0.60	0.11	−0.59	0.55	−0.28	0.15	None
						Combined	0.09	0.09	1.00	0.32	−0.08	0.27	None
	buffy	46	41	−6.44	0.54	Exposure	−0.09	2.21	−0.04	0.96	−4.25	4.43	None
						Flight	<−0.01	3.15	<0.01	0.99	−6.19	6.18	None
						Combined	0.04	2.44	0.02	0.98	−4.74	4.83	None
	hbg3	46	42	−124.66	54.33	Exposure	0.07	0.04	1.62	0.11	−0.15	0.15	None
						Flight	1.39	0.03	3.17	**<0.001**	1.34	1.46	**Up**
						Combined	−0.74	0.05	31.23	**<0.001**	−0.85	−0.64	**Up**
Major royal jelly proteins	*mrjp1*	46	41	−242.23	10.74	Exposure	−16.32	21.08	−0.77	0.44	−57.64	24.98	None
						Flight	39.01	29.77	1.31	0.19	−19.35	97.36	None
						Combined	8.22	23.15	0.36	0.72	−37.14	53.59	None
	mrjp2	46	41	−228.29	10.14	Exposure	−22.86	16.79	−1.36	0.17	−55.78	10.05	None
						Flight	−6.73	22.84	−0.29	0.77	−51.49	38.03	None
						Combined	−12.36	18.47	−0.67	0.51	−48.56	23.85	None
	mrjp3	48	43	−122.25	5.30	Exposure	−3.14	0.46	−6.80	**<0.001**	−4.05	−2.24	**Down**
						Flight	−2.04	0.43	−4.73	**<0.001**	−2.89	−1.19	**Down**
						Combined	−3.22	0.49	−6.51	**<0.001**	−4.18	−2.25	**Down**

(95 % CI 5.8, 7.7 km) for control and exposed bees, respectively. Subsequently, thiamethoxam-exposed bees flew 25 % further when compared to the controls. There was no influence of body mass on flight distance ($z = -1.191$, $P > 0.05$). Further, thiamethoxam exposure led to faster average flight speeds (*meglm*; $z = 1.98$, $P = 0.047$; Fig. 1C), where average flight speed was 2.54 ± 0.27 m s^{-1} and 2.47 ± 0.26 m s^{-1} for thiamethoxam-exposed and controls, respectively (mean ± SD). In contrast, thiamethoxam-exposure had no significant effect on maximum flight speed (*meglm*; $z = -0.09$, $P = 0.93$), with the mean maximum flight speed across both treatments being 4.93 ± 0.33 [m s^{-1}].

3.2. Molecular transcript expression: oxidative phosphorylation cytochrome genes

Thiamethoxam exposure, flight activity and the interaction between both factors revealed no significant effect on *cox6c* transcript expression (all z's < 0.61.; all P's > 0.21; Table 1). In contrast, thiamethoxam exposure and flight activity both had a significant negative effect on *cox5a* and *cox17* transcript expression (all z's < −1.99; all P's < 0.05; Table 1). Further, a significant interaction was observed between the two factors (both z's < −5.06; both P's < 0.001; Table 1); resulting in a significant down regulation of *cox5a* and *cox17* transcripts by 1.9- and 2.2-fold, respectively (Table 1, Fig. 2A & B). In contrast, while *cox5b* was not affected by thiamethoxam exposure alone ($z = 1.36$; $P = 0.17$), both flight activity and the interaction between exposure and flight revealed a significant negative effect on the transcript expression (both z's < −2.14; both P's < 0.03; Table 1). Hedges' d values for *cox5a*, *cox5b* and *cox17* were 0.06 ± 0.13, −0.22 ± 0.12, and 0.09 ± 0.13, respectively (mean [Hedge's d] ± 95 % CI; Fig. 2C); suggesting an additive effect for *cox5a* and *cox17* and an antagonistic effect for *cox5b*.

3.3. Endocrine regulation genes

Thiamethoxam exposure did not significantly affect the expression levels of *vg*, *ilp-1*, *buffy* or *hbg3* (*meglm*; all z's < 0.74; all P's > 0.37, Table 1, Fig. 2A & B). Likewise, flight activity did not significantly affect the transcript expression levels of *vg*, *ilp-1* or *buffy* (all z's < 1.0; all P's > 0.32). However, the expression of *hbg3* was significantly up-regulated ($z = 3.17$; $P < 0.001$), resulting in a 1.58-fold increase compared to the control. Furthermore, the only significant interaction term was for *hbg3* ($z = 31.23$; $P < 0.001$), where an up-regulation of the expression was observed compared to controls (Fig. 2A & B). The Hedges' d value for *hbg3* was 0.21 ± 0.13, revealing a synergistic interaction (mean [d] ± 95 % CI; Fig. 2C).

3.4. Royal jelly protein genes

Neither thiamethoxam exposure (z's < 0.77; both P's > 0.17), flight activity (z's < 1.31; P's > 0.19), nor the interaction term (z's < 0.36; both P's > 0.51) had a significant effect on the expression of *mrjp1* and *mrjp2* (*melgm*; Table 1, Fig. 2A & B). In contrast, thiamethoxam exposure ($z = -6.80$; $P < 0.001$) and flight activity ($z = -4.73$; $P < 0.001$) significantly down-regulated the expression of *mrjp3* (*meglm*; Table 1; Fig. 2A & B), with the Hedges' d value being 0.40 ± 0.13, thus reflecting a synergistic interaction (mean [d] ± 95 % CI; Fig. 2C).

3.5. Flight and expression correlations

A significant negative correlation was observed between *cox5a* expression and total flight distance ($t = -3.6$; $P = 0.007$; Fig. 3). The remaining genes did not significantly correlate with total flight distance (all P's > 0.05). In addition, *cox5a*, *cox5b* and *cox6c* all revealed a significant negative correlation with total flight duration (all t's < −2.62; P < 0.011; Fig. 3), whereas the remaining genes showed no significant

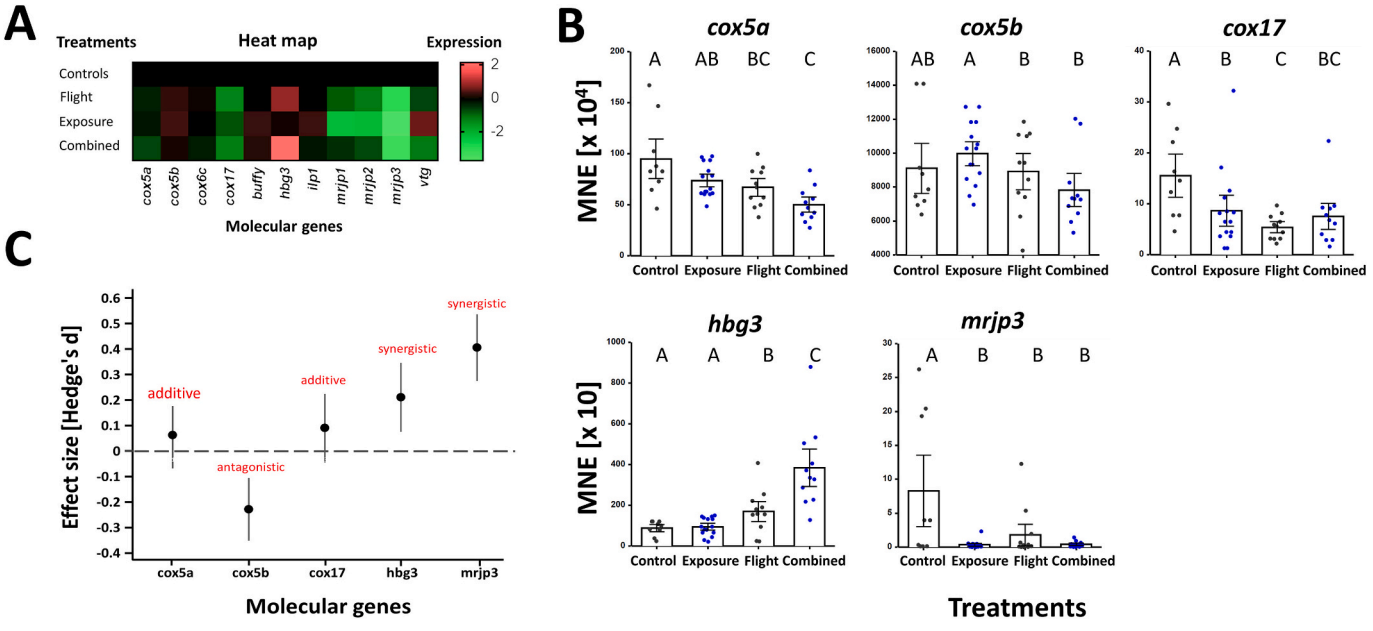

Fig. 2. Molecular gene and interaction assessment of female (worker) honey bees, *Apis mellifera*, exposed to the neonicotinoid insecticide thiamethoxam before and after tethered flight mill assessment. (A) Heat map showing up- and downregulation of molecular transcripts relative to the controls. Red shading indicates an upregulation whereas green reflects a downregulation of the molecular transcript and black indicates no change in expression. (B) Bar charts showing the mean normalized expression (NME) of molecular transcripts. All bar charts show medians and 95 % Confidence Intervals; dots represent individual measurements. Significant differences amongst treatment groups are indicated by different capital letters (Bonferroni multiple-pairwise comparison test: $P < 0.05$). (C) Overview of the interactions determined between the two tested factors (i.e., flight and insecticide exposure).

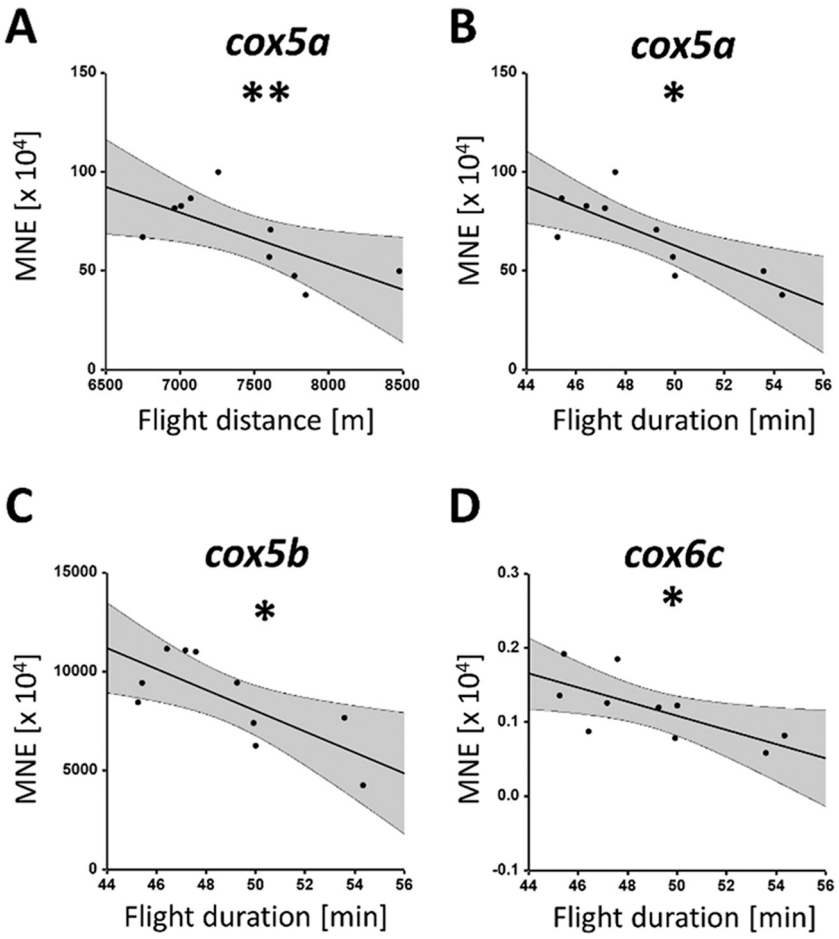

Fig. 3. Correlations between flight behavior (i.e., distance and duration) and mean normalized expression (MNE) of *cox* genes in control female (worker) honey bees, *Apis mellifera*. A significant negative correlation was observed for NME of three *cox* genes in relation with flight distance (A) and flight duration (B, C, and D). A significant negative correlation between NME and flight behavior is indicated by $^{*}P < 0.05$. Black dots represent individual specimen, whereas the black lines and the shaded area represent the regression line as well as its 95 % confidence intervals.

correlation (all *P*'s > 0.05). Lastly, none of the assessed gene expression levels correlated with mean or maximum flight speed (all *P*'s > 0.25).

4. Discussion

Our results demonstrate that acute exposure to an environmentally relevant dosage of thiamethoxam can alter flight behavior and elicit molecular dysregulation in honey bee foragers. Neonicotinoid exposure led to bees flying further and faster, likely explained by the insecticide acting upon the nervous system and causing hyper-activity (Gill and Raine, 2014; Tosi and Nieh, 2017). In addition, the data confirm that thiamethoxam can alter molecular transcription profiles - particularly transcripts associated with the oxidative phosphorylation process, which suggests that thiamethoxam may interfere with mitochondrial ATP synthesis. Interestingly, thiamethoxam-induced alterations on the transcription profiles were more pronounced when in combination with flight activity, highlighting that neonicotinoid effects are more likely to be revealed when bees are simultaneously undertaking physical activities (e.g., flight). Our results underscore that genes involved in the oxidative phosphorylation process may be promising biomarkers to determine potential inadvertent effects of xenobiotic exposure in bees (Lu et al., 2020; Christen, 2023), yet additional data are required to deepen our understanding of potential links.

Control foragers flew an average distance of 5.7 km, which lies within the range reported by previous field studies (2.3–6.1 km; e.g., Visscher and Seeley, 1982; Beekman and Ratnieks, 2000). Previous tethered flight mill studies using honey bee foragers revealed shorter flight distances (e.g., between 1.8 and 3.0 km) (Brodschneider et al., 2009; Blanken et al., 2015; Wells et al., 2016; Tosi et al., 2017), which may be attributed to variations in experimental design, such as the use of pollen foragers of an unknown age (this study), compared to a specific age-cohort (e.g., 20 days (Brodschneider et al., 2009)). In addition, the duration that specimens were kept under laboratory conditions prior to being placed on the tethered mills (i.e., hours in this study vs. days in Brodschneider et al. (2022)), as well as whether they first performed an exhaustion flight (e.g., Wells et al. (2016)) and varying flight mill apparatus may also explain the differences in flight performance across studies. In line with previous studies (Tosi et al., 2017; Ma et al., 2019), our data confirm that acute exposure to thiamethoxam altered the flight performance of honey bee foragers. The increased flight duration, distance, and speed are most likely attributed to neural hyper-excitation, triggered by the neonicotinoid acting on the nervous system (Tomizawa, 2013). Likewise, hyper-excitation may explain the increased motivation (i.e., willingness) of exposed individuals to fly on the mills, compared to controls. The acute hyper-activity and increased flight duration observed will inevitably cause premature depletion of energy and muscular exhaustion (Tosi et al., 2017; Kenna et al., 2019). Altered flight performance, coupled with known negative effects of neonicotinoids on cognitive abilities (Stanley et al., 2016; Siviter et al., 2018), are likely to lead to impaired foraging efficiency (Colin et al., 2019; Ma et al., 2019; Christen et al., 2021). Furthermore, considering environmental contamination of neonicotinoids is almost ubiquitous

(Zioga et al., 2020; Woodcock et al., 2021), bees are more likely to face chronic rather than acute exposure when foraging. Previous studies have revealed that chronic neonicotinoid exposure can lead to hypoactivity, causing longer phases of inactivity, reduced flight endurance, and impaired motor function, which may further impair foraging ability (Williamson et al., 2014; Tosi et al., 2017; Tosi and Nieh, 2017; Crall et al., 2018). In light of the importance of forager efficiency in maintaining colony functionality and ultimately colony fitness (Raine and Chittka, 2008; Henry et al., 2015), inadvertent effects of neonicotinoid exposure on honey bee foragers may cause time-lag effects that contribute to increased colony dysfunction and ultimately colony failure. However, recent higher-tier studies exposing honey bee colonies to neonicotinoids under field conditions do not necessarily support this notion (Henry et al., 2015; Rundlöf et al., 2015; Thompson et al., 2019). This is likely attributed to the ability of eusocial insects to buffer against environmental stressors at the colony level (Straub et al., 2015). Nevertheless, additional long-term data are required that ideally test the impact of neonicotinoid exposure across varying timepoints (i.e., seasons) and across multiple years (Woodcock et al., 2017; Schläppi et al., 2020).

The molecular data revealed that the transcription profiles were significantly altered by thiamethoxam exposure and flight activity, individually and in combination. In line with previous studies (e.g., Margotta et al., 2012; Christen et al., 2021), our data show that both flight and thiamethoxam exposure downregulated *cox*-transcripts that are closely associated with oxidative phosphorylation and ATP production (Mao et al., 2017). In addition, we revealed a novel negative correlation between flight performance (i.e., duration and distance) and *cox* transcript expression levels. This result is somewhat counterintuitive, as we might anticipate higher cox-transcript levels in response to energetically demanding, long lasting flight. However, lower *cox* transcript expression may convey that flight activity in insects incurs a cost, possibly associated with increased oxidative damage to proteins and lipids (Magwere et al., 2006), causing impaired mitochondrial functionality and reduced gene expression. However, further studies are warranted to confirm this assumption. Considering that flight alone may alter the efficiency of oxidative phosphorylation processes, potential inadvertent effects of neonicotinoids on *cox*-transcripts may represent an additional burden on the flight abilities of bees. Indeed, flight in combination with exposure caused a significant additive interaction, which led to a reduction in *cox5a* and *cox17* expression. While our results contradict those of Fent et al. (2020), which revealed that *cox17* was upregulated post neonicotinoid exposure, a downregulation of *cox*-transcripts has been reported for honey bees exposed to fungicides, which led to reduced ATP productivity in flight muscles (Mao et al., 2017). Moreover, considering that neonicotinoids can reduce energy metabolism and mitochondrial functionality in bees (Nicodemo et al., 2014; Moffat et al., 2015; Powner et al., 2016; Syromyatnikov et al., 2020), our results provide a plausible mechanistic explanation for impaired flight endurance in chronically exposed bees (Tosi et al., 2017; Kenna et al., 2019). However, acute and chronic dose-response tests are required to confirm this.

Aligned with previous research (e.g., Christen et al., 2016; Fent et al., 2020), our data confirm that endocrine system related genes can be altered by field-relevant dosages of neonicotinoids, as seen in the downregulation of *mrjp3*. Elucidating the biological meaning of this result remains complex, but the fact that expression in hormone-associated transcripts was changed is of critical relevance. This not only holds true for foragers but also nurse bees within the hive as they are also frequently exposed to neonicotinoid contaminated hive material (i.e., wax, pollen, and honey) (Xiao et al., 2022; Schaad et al., 2023). Hormone expression levels in the brain and hypopharyngeal glands determine age-development and behavioral traits of workers and forager bees (Fent et al., 2020). Alterations in the expression profile due to neonicotinoid exposure may affect development and behavior of workers, which could lead to precocious foragers or foragers to revert to nursing. Precocious foragers tend to be less efficient and are also prone to higher risk of dying during their initial flights (Requier et al., 2020). Furthermore, *mrjp*'s are expressed in Kenyon cells, which are involved in associative learning and memory within the mushroom bodies of bees (Kucharski et al., 1998), and changes in *mrjp* expression may alter the navigational abilities of foragers. Ultimately, inefficient or fewer foragers would inevitably disrupt colony function and increase chances of colony failure. While studies suggest that such adverse effects can be tolerated by honey bee colonies (e.g., Cutler et al., 2014; Siede et al., 2017; Thompson et al., 2019), this may not be the case for other eusocial (e.g., bumble bees) and solitary bee species (Rundlöf et al., 2015), due to the lack of colony level buffering capacities (Straub et al., 2015). Lastly, while thiamethoxam alone revealed no significant effect on *hbg3* expression, when combined with flight activity the expression level was significantly upregulated, revealing a synergistic interaction. This result emphasizes that pesticide effects can vary depending on whether the bees are conducting arduous tasks (e.g., flight) or not, calling for more thorough ecotoxicological studies at the field level. Therefore, we welcome the incorporation of additional data from novel higher-tier semi-field and field studies (e.g., homing flight assessments using RFID technology (OECD, 2021)) to prevent possible false negative results in environmental risk assessments (Straub et al., 2020).

Our study confirms that acute neonicotinoid exposure can alter flight behavior as well as transcription profiles in honey bee workers. Furthermore, our experiment is amongst the first to link behavioral flight traits with molecular expression data, suggesting that a dysfunction in oxidative phosphorylation processes may underlie the altered flight behavior. An improved mechanistic understanding of how xenobiotics affect bees at the individual level will undoubtedly help mitigate ongoing losses of managed honey bees and declines in wild insect populations. Similar tethered flight mill studies on wild bees, in particular solitary species that cannot rely on the environmental buffering capacities such as eusocial insects (Straub et al., 2015) may provide interesting results and could offer a plausible mechanistic explanation for impaired foraging capacities and reduced fitness in wild bees (Rundlöf et al., 2015; Knauer et al., 2022; Albacete et al., 2024).

CRediT authorship contribution statement

Verena Christen: Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lukas Jeker:** Writing – review & editing, Resources, Methodology, Investigation. **Ka S. Lim:** Writing – review & editing, Software, Methodology, Formal analysis, Conceptualization. **Myles H.M. Menz:** Writing – review & editing, Resources, Methodology, Conceptualization. **Lars Straub:** Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.177166.

Data availability

The complete raw data can be found at the Dryad repository. See: https://doi.org/10.5061/dryad.4j0zpc8mh.

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