Eusocial insect declines: Insecticide impairs sperm and feeding glands in bumblebees

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HIGHLIGHTS

- Reduced bumblebee colony fitness due to agrochemicals is poorly understood.
- Sperm traits and feeding glands of bumblebees were measured post-thiamethoxam exposure.
- Survival was not affected, but both sperm and feeding gland quality were impaired.
- The data provide plausible mechanistic explanations for recent bumblebee declines.

GRAPHICAL ABSTRACT
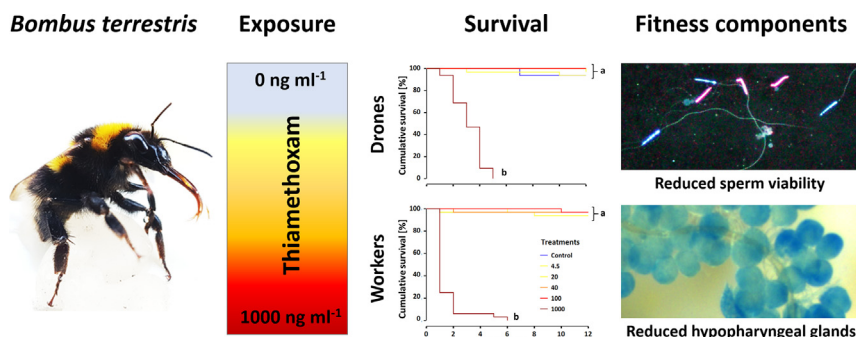
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ABSTRACT

Insecticides are contributing to global insect declines, thereby creating demand to understand the mechanisms underlying reduced fitness. In the eusocial Hymenoptera, inclusive fitness depends on successful mating of male sexuals (drones) and efficient collaborative brood care by female workers. Therefore, sublethal insecticide effects on sperm and glands used in larval feeding (hypopharyngeal glands (HPG)) would provide key mechanisms for population declines in eusocial insects. However, while negative impacts for bumblebee colony fitness have been documented, the effects of insecticide exposure on individual physiology are less well understood. Here, we show that field-realistic concentrations (4.5–40 ng ml^{-1}) of the neonicotinoid insecticide thiamethoxam significantly impair _Bombus terrestris_ sperm and HPGs, thereby providing plausible mechanisms underlying bumblebee population decline. In the laboratory, drones and workers were exposed to five thiamethoxam concentrations (4.5 to 1000 ng ml^{-1}). Then, survival, food consumption, body mass, HPG development, sperm quantity and viability were assessed. At all concentrations, drones were more exposed than workers due to higher food consumption. Increased body mass was observed in drones starting at 20 ng ml^{-1} and in workers at 100 ng ml^{-1}. Furthermore, environmentally realistic concentrations (4.5–40 ng ml^{-1}) did not significantly affect survival or consumption for either sex. However, thiamethoxam exposure significantly negatively affected both sperm viability and HPG development at all tested concentrations. Therefore, the results indicate a trade-off between survival and fitness components, possibly due to costly detoxification. Since sperm and HPG are corner stones of colony fitness, the data offer plausible mechanisms for bumblebee population declines. To adequately mitigate ongoing biodiversity declines for the eusocial insects, this study suggests it is essential to evaluate the impact of insecticides on fitness parameters of both sexuals and workers.

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1. Introduction

Biodiversity losses are critical for conservation because changes to ecosystems inevitably impact human society (Eisenhauer et al., 2019). Recent reports of insect declines are particularly concerning considering their indispensable role as key providers of ecosystem services (Wagner, 2020). These alarming declines are often closely associated with a multitude of anthropogenic factors including climate change (Soroye et al., 2020), invasive species (Turbelin et al., 2017), habitat loss and fragmentation (Dirzo et al., 2014), as well as increased use of agrochemicals (Seibold et al., 2019). The ubiquitous use of agrochemicals, especially neonicotinoid insecticides, has received considerable attention as a major factor contributing to insect population declines (Woodcock et al., 2016; Schläppi et al., 2020; Warren et al., 2021). Despite temporary and partial bans of certain neonicotinoids in parts of Europe and other regions of the world, they still make up to ~25% of the global market (Douglas and Tooker, 2015). While not only being widely used for agricultural purposes (Chen et al., 2019; DiBartolomeis et al., 2019), they are frequently also used in forested areas, garden centers and nurseries, as well as in biocides for home garden applications or pet medicines (Cloyd and Bethke, 2011; Cowles, 2009; Lentola et al., 2017). While this has prompted extensive research efforts, the mechanisms underlying the impact of neonicotinoids on fitness in non-target insects is still poorly understood.

Neonicotinoids and their metabolites are highly effective in disrupting neural transmission by binding to acetylcholine receptors in the central nervous system of insects (Matsuda et al., 2020). Due to their systemic nature, high water solubility, and persistence, widespread neonicotinoid contaminations can accumulate and remain in the environment over a long period (Douglas et al., 2020; Humann-Guilleminot et al., 2019). This has become problematic for pollinators, as they can be exposed via contaminated pollen, nectar, or guttation-fluids of numerous flowering crops and wildflowers, (Botías et al., 2016; David et al., 2016; Wintermantel et al., 2020), as well as water (Borsuah et al., 2020). The duration and magnitude of neonicotinoid exposure can vary depending on the application method, season, country, or landscape (Jones et al., 2014; Byrne et al., 2014; Woodcock et al., 2021, 2017), with field-realistic exposure ranging anywhere below 1 to beyond 40 ng ml^{-1} for several days to weeks (Calvo-Agudo et al., 2019; el Agrebi et al., 2020; McArt et al., 2017; Stoner and Eitzer, 2012). Consequently, ample studies have revealed lethal and sublethal effects of neonicotinoid exposure on pollinating insects (Lu et al., 2020), in particular for bee species (Hopwood et al., 2016).

While individual lethality in bees is seldom the outcome of chronic field-realistic exposure to neonicotinoid insecticides (Neumann et al., 2015), many sublethal impacts have been reported in both managed and wild species, including bumblebees (Mommaerts and Smagghe, 2011). These effects can have negative consequences on behavior (e.g., orientation, thermoregulation, learning or feeding (Crall et al., 2018; Muth et al., 2020; Stanley et al., 2015)), physiological traits (e.g., fecundity, development, immune response, brain growth or body mass (Baron et al., 2017b; Dance et al., 2017; Smith et al., 2020; Wu-Smart and Spivak, 2016)), endocrine system (Baines et al., 2017), as well as colony development (e.g., production or size of sexuals, egg-laying or colony founding (Arce et al., 2017; Ellis et al., 2017; Whitehorn et al., 2012)). However, identifying individual physiological traits that may explain reduced colony fitness and ultimately the ongoing declines of bumblebee and other eusocial insect populations remains challenging.

Bumblebees, such as *Bombus terrestris*, are ideal model species to study the consequences of environmental stress on fitness, as they can be maintained under laboratory and (semi-)field conditions. Fundamentally, bumblebee colony fitness can be defined as the number of successfully mated male sexuals (drones; = male fitness) as well as the number of female gynes (future queens) that will successfully establish a new colony (= female fitness). This is straightforward to

estimate, both in the field (Ellis et al., 2017) as well as in the laboratory (Whitehorn et al., 2012). However, the underlying mechanisms for colony fitness in bumblebees are far more difficult to pinpoint compared to a solitary species, because eusocial insects exhibit reproductive division of labor, overlapping generations, and cooperative brood care. The worker caste usually gains inclusive fitness by providing colony functionality via foraging, thermoregulation, and brood care, which is essential for the eventual production of male and female sexuals (Moritz and Southwick, 2012; but also see laying workers (Neumann and Moritz, 2002; Lopez-Vaamonde et al., 2004)). Previous studies have shown that neonicotinoids can reduce colony growth and the number of sexuals produced (Arce et al., 2017; Ellis et al., 2017; Whitehorn et al., 2012; Woodcock et al., 2017), possibly due to impaired worker efficiency and functionality (i.e., reduced foraging, nursing, or thermoregulation) (Crall et al., 2018; Gill et al., 2012). However, evidence of impaired bumblebee worker physiology essential for optimal brood care (i.e., glands used in larval feeding; hereafter hypopharyngeal gland (HPG)), is lacking. Although the function of bumblebee HPGs are not yet fully understood, they are known to be essential for digestion (i.e., via enzymes such as amylase and invertase) and endocrine regulation during nutritional uptake, as well as storing vitellogenin (Jedlicka et al., 2016; Pereboom, 2000). Neonicotinoid exposure has been associated with a reduced HPG acini width in honeybees (Hatjina et al., 2013; Renzi et al., 2016), which may be responsible for the impact on nutritional composition of brood food (Milone et al., 2021). Given that similar effects are shown in bumblebees, this could offer a mechanistic explanation for observed impacts on colony fitness.

The male fitness of bumblebee colonies obviously depends on optimal reproductive capacities of drones (e.g., mating behavior or physiology), especially due to the monandrous mating system. Indeed, with a few exceptions (e.g., *Bombus hypnorum* (Brown et al., 2002)), most bumblebee species display monandry, where gynes rely on a single successful mating. Even though the role of bumblebee drones is clearly indispensable, the impact of xenobiotics on their reproductive physiology (e.g., sperm quantity and viability) remain unknown, despite negative effects being observed for honeybees (Ciereszko et al., 2017; Straub et al., 2016). This constitutes a major knowledge gap for bumblebees as mating with drones possessing reduced sperm quantity and viability may have severe consequences for colony development. For instance, impaired sperm viability may result in unintentional male-biased sex-ratios in haplo-diploid species, where males usually develop from unfertilized eggs and females from fertilized ones (Beye et al., 2003). This could be especially important during the initial nest founding stage. Therefore, sperm quantity and viability reflect fitness components that may provide a previously overlooked explanation for population declines if negatively affected.

Here, we investigated the lethal and sublethal effects (i.e., consumption, body mass difference, HPG acini width, and sperm traits) of chronic neonicotinoid insecticide exposure on individual adult worker and drone bumblebees, *B. terrestris*, to explore mechanistic explanations for reports of reduced colony fitness and population declines. Therefore, a laboratory dose-response test was applied, with exposure scenarios ranging from 0 to 1000 ng ml^{-1}. Maintaining bees individually enabled a precise measurement of food consumption, thereby shedding light on potential neonicotinoid-induced differences within and between the sexes, resulting in varying exposure scenarios. We hypothesized that field-realistic dosages would not significantly impact survival and consumption, in line with previous studies (Laycock et al., 2014; Mommaerts et al., 2010), but would significantly negatively affect fitness components (i.e., HPG acini width and sperm traits) as in honeybees (Renzi et al., 2016; Ciereszko et al., 2017; Straub et al., 2016).

2. Material and methods

The experiment was conducted between May and December 2019 at the Institute of Bee Health, University of Bern, Switzerland and the

Department of Entomology and Plant Pathology, Auburn University, USA. To establish known age-cohorts, a total of 448 ($N_{drones} = 224$; $N_{workers} = 224$) newly emerged *Bombus terrestris* drones and workers were randomly collected from over 50 different colonies by Biobest Group NV®, Belgium. Based on their physical appearance, bumblebees aged 0–24 h typically have a silvery grey coloration indicating recent eclosion (Alford, 1975). Due to shipping duration to Switzerland, the age of the bees upon arrival was no more than three days and visual inspections revealed that all individuals were free of ectoparasite infestations, clinical symptoms of disease or other abnormalities (Goulson, 2003). The chronic dose-response test as well as the sperm assessments were conducted in Bern, Switzerland, whereas the heads of the workers were sent to Auburn, USA, for analyses of the HPGs.

2.1. Set-up

To determine *B. terrestris* drone and worker sensitivity to thiamethoxam, a chronic dose-response test was conducted. All individuals ($N_{drones} = 224$; $N_{workers} = 224$) were placed in separate cages [100 cm³] (Williams et al., 2013) and maintained at 28 °C and 60% relative humidity in complete darkness (OECD, 2017a). Each bee was given 50% [w/w] sucrose-solution *ad libitum* via a 5 ml syringe to provide sufficient carbohydrates (van der Steen, 2001). Syringes were weighed and exchanged every four days to measure consumption and exposure, as well as to prevent possible fungus contamination. Before being assigned to a treatment group, the initial body mass of each experimental bee was recorded to the nearest 0.1 mg using an analytic scale (Mettler Toledo AT400). Individuals were randomly allocated to a treatment group: Controls, or one of five tested thiamethoxam concentrations (4.5, 20, 40, 100, 1000 ng ml⁻¹) ($N_{drones} = 32$ and $N_{workers} = 32$ per dose). The suggested thiamethoxam LC50 value is 33 ng bee⁻¹ (120 ng ml⁻¹); however, this was based on small queenless colonies (i.e., microcolonies) (Mommaerts and Smagghe, 2011). We consider 4.5 to 40 ng ml⁻¹ to be field-realistic doses of thiamethoxam, as concentrations in nectar and honeydew have been found within this range (Calvo-Agudo et al., 2019; Sanchez-Bayo and Goka, 2014). Further, soil nesting species, such as *B. terrestris*, may even encounter exposure levels well beyond 40 ng ml⁻¹ and in rare cases even up to 100 ng ml⁻¹ (Girolami et al., 2009; Goulson, 2013; Reetz et al., 2016) due to soil contaminations. The 1000 ng ml⁻¹ was used as a positive control and does not reflect a field-realistic exposure scenario. Bumblebees that have survived 12 days were used for sperm or hypopharyngeal gland (HPG) analysis, respectively.

2.2. Chronic oral toxicity test and insecticide solution preparation

A modified chronic oral toxicity test was performed following the OECD 247 and 245 test guidelines for bumblebee acute oral toxicity and honeybee chronic oral toxicity, respectively (OECD, 2017a, 2017b). In contrast to the OECD 245 test guideline, the chronic exposure lasted 12 instead of ten days, to ensure drones were exposed throughout sexual maturity, as *B. terrestris* drones naturally mate at the age of 12 days onwards and maximum sperm migration to the vasa deferentia and seminal vesicals occurs on day 13 (Tasei et al., 1998). Furthermore, while the size of bumblebee HPGs can remain consistent until day 50 depending on colony task, they are usually fully developed after 13 days (Röseler, 1967). Additionally, to minimize interference, the sucrose-solution feeders were replaced every four days instead of daily, as the degradation of thiamethoxam under non-UV-exposure is likely to be negligible (Fryday et al., 2015; Schläppi et al., 2020). Pure analytical grade thiamethoxam (Sigma Aldrich 37,924-100MG-R, UK) was dissolved in a known volume of distilled water to produce a primary stock solution (1 mg ml⁻¹) and maintained at 4 °C in a fridge to prevent UV-light degradation. To ensure complete dilution of the chemical, acetone was added as a dissolvent which accounted for less than 0.5% of the volume in the final thiamethoxam sucrose-solutions. An aliquot of the primary stock solution was then used to produce a secondary solution

with a 10 µg L 1 (10 ng ml⁻¹) thiamethoxam concentration before each feeding. Varying volumes of the secondary solution were added to a 1:1 sucrose/water [w/w] solution to produce the first four desired concentrations (i.e., 4.5–100 ng ml⁻¹). Acetone was also added to the sucrose-solution to account for potential negative effects of the dissolvent.

2.3. Consumption, exposure, survival, and body mass

Sucrose consumption was measured by recording the mass of the syringe every four days until the experiment was terminated or at the point of death. In addition, evaporation was measured by placing syringes in empty cages, but the results revealed that evaporation was below 1% and thus negligible. Total consumption [g] was divided by the number of days that the bee was alive to obtain an average daily consumption [g day⁻¹]. Daily and total consumption was then multiplied by the exposure concentration to calculate daily [ng day⁻¹] and total [ng] thiamethoxam exposure for each individual bee, respectively. Survival was recorded every 24 h. The body mass of the individuals that survived 12 days was recorded and subtracted from their initial mass to calculate body mass difference post-experiment. Body mass of dead bees was not recorded due to post-mortem desiccation.

2.4. Sperm quantity and viability

All surviving drones (N = 169) were assessed for sperm quantity and viability 12 days post-cage assay initiation. Subsequently, drones were between 14 and 15 days of age and considered sexually mature. Individuals were briefly anaesthetized using CO_2 before being pinned to a wax plate and dissected. Sperm samples were collected from live bees following (Baer and Schmid-Hempel, 2000); however, the entire drone genitalia, including the granular gland, accessory gland, vesical seminalis and testis were removed from each drone (Fig. 1A), placed in a 1.5 ml Eppendorf® tube containing 200 µl Kiev+ buffer, and gently crushed to form a diluted sperm stock solution. Immediately after, a 50 µl aliquot of the sperm stock solution was set aside in a separate 1.5 ml Eppendorf® tube for analyses of sperm viability (proportion of sperm alive). Sperm viability was quantified according to (Wegener et al., 2012). In brief, each sample was diluted with 50 µl of Kiev+ buffer before 1 µl of propidium iodide (PI) solution (1 mg ml⁻¹) and 0.5 µl of Hoechst 33342 (0.5 mg ml⁻¹) (both Sigma-Aldrich, UK) were added to the suspension. Samples were then incubated for ~20 min in complete darkness and then gently vortexed. Ten µl were viewed at 400× magnification using fluorescent microscopy (Olympus BX41, Switzerland) equipped with filter cubes for UV excitation (Fig. 1B). Ten visual fields were randomly selected for each sample to quantify living and dead sperm and an average value was then calculated upon these fields (Wegener et al., 2012). Sperm counts were performed by adding 50 µl of stock sperm solution diluted in 50 µl Kiev+ buffer (1:1 dilution) in a 1.5 ml Eppendorf® tube. The sperm density was then measured using a Neubauer counting chamber under light microscopy (Thermo Fischer Scientific, USA). The final sperm density was quantified by applying the following calculation (Strobl et al., 2019): Total sperm quantity (200 µl) = average number of sperm counted in two Neubauer counting chambers x conversion factor (50,000)/(200 µl/1000 µl).

2.5. Hypopharyngeal glands

Workers alive at day 12 (i.e., age 14–15) were used for the HPG assessment (N = 133). Therefore, workers were briefly anaesthetized using CO_2 before the heads were dissected. Heads were shipped to the USA in separate 2 ml Eppendorf® tubes containing 0.5 ml of 2% paraformaldehyde PBS preservation buffer (Lanier and Warner, 1981), and kept at 4 °C until the HPGs were dissected according to (Carreck et al., 2013). In brief, each head was air-dried for 5 min after removal from their tubes. Once dry, the head was glued individually at the posterior end onto a wooden dissection block (Fig. 1C); antennae were then
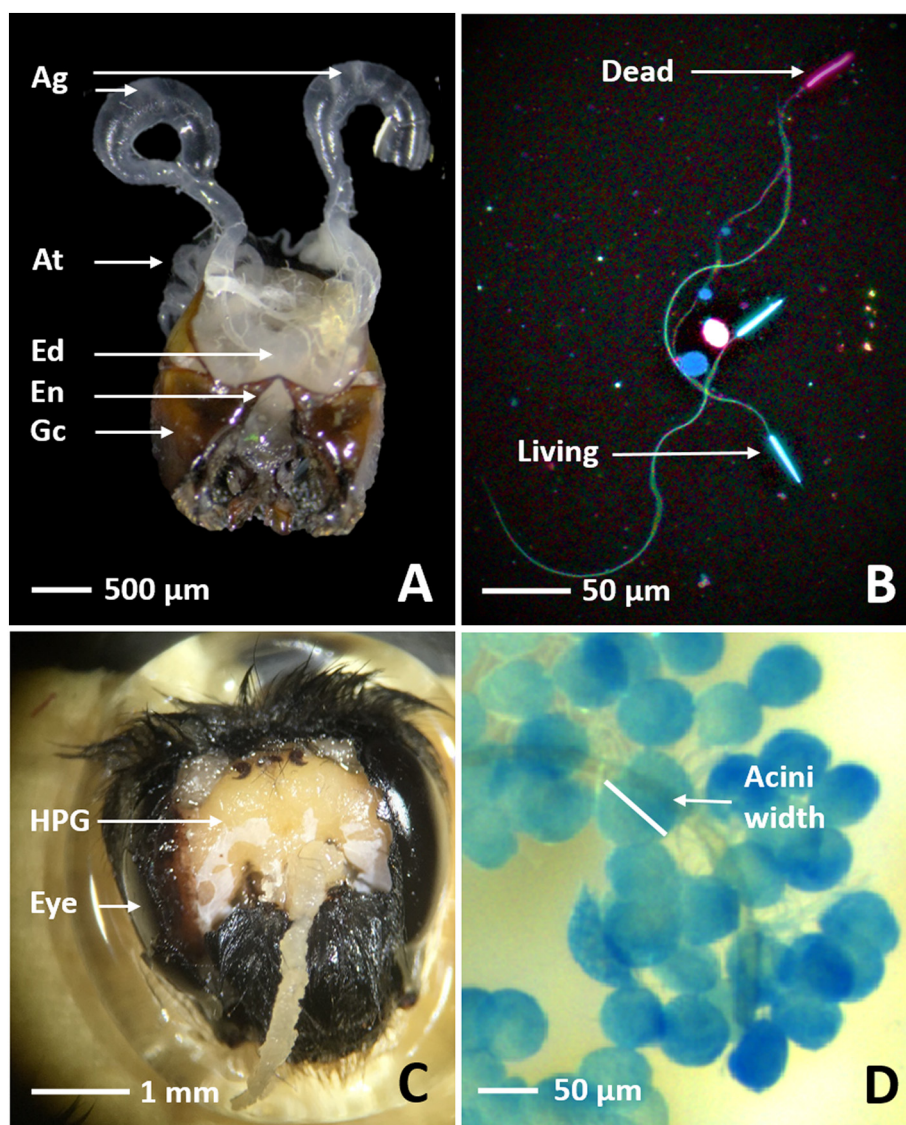
Fig. 1. *Bombus terrestris* male genitalia and sperm and worker hypopharyngeal glands and acini. (A) Reproductive tract from drones showing accessory glands (Ag), accessory testes (At), ejaculatory duct (Ed), endophallus (En), and the gonocoxa (Gc). Sperm was taken from entire reproductive tract for analysis. (B) Fluorescence stained sperm used for viability analysis, where the head of living sperm cells are blue and the head of dead sperm cells is colored red. (C) Head of a worker, with the fons removed to expose the hypopharyngeal glands (HPG). (D) Slide of mounted HPG acini under 5× compound light microscopy for measurement in ImageJ.

removed at their junctions. The frons was lifted off after a cut was made across the ocelli, along the margins of the compound eyes and the mask, but excluding clypeus, labrum and mandibles. The exposed HPGs were then removed using forceps and deposited in a petri dish containing 0.5 ml saline dissection buffer for 5 min. Single acini were accentuated by adding 0.5 ml of Coomassie Brilliant Blue g-250 stain (Hartfelder et al., 2013), which was dispensed throughout the extracted glands by gently shaking the petri dish. After 5 min, the glands were mounted on a wetted glass slide using a coverslip. Slide mounted acini were examined using a 5× compound light microscopy (Olympus BX41) and digital microscope photography (Olympus DP72) (Fig. 1D). Thirty acini diameters per individual were measured perpendicular to their attachment point with the imaging software ImageJ 1.x using a 50 μm as a measurement scale (Schneider et al., 2012).

2.6. Statistical analyses

All statistical analyses were performed using STATA16 (StataCorp, 2017), whereas figures were created using NCSS20 (NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss). The Shapiro-Wilk's and the Levene's test were used to test data and model residues for normal distribution homogeneity of variances and choose statistical tests accordingly. To assess the relationship between the explanatory variable exposure [ng day^{-1}] and the dependent variables (i.e., body mass difference, sperm and HPG traits) linear regression was applied using the function *regress*, where individual bees were considered independent units and initial mass was included as an additional explanatory term. Additionally, to determine differences between treatments, multilevel generalized logistic or linear (regression) models (GLMMs) and general linear models (GLM) with random intercepts were fit using the functions *meglm* and *glm*. Individual bees were considered independent units; treatments (insecticide vs control), sex and initial mass were included as the explanatory (fixed) terms. For HPG analysis, individual was incorporated as a random effect because there were multiple measures per bee. For each model, a stepwise backward elimination approach was applied to determine the model of best fit. 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. Post-hoc tests were performed by using the multiple pairwise comparisons (Bonferroni

Table 1

Summary of individual consumption and exposure results for *Bombus terrestris* drones and workers exposed to different concentrations of thiamethoxam. For each treatment and sex, the daily and total values for consumption as well as exposure are provided (median ± 95% CI). Total values only include individuals that survived the 12 day experiment.

Treatments [ng ml^{-1}]	Sex	Individual consumption		Individual exposure	
		Daily consumption [g day^{-1}]	Total consumption [g]	Daily exposure [ng day^{-1}]	Total exposure [ng]
0	Drones	0.293 ± 0.261–0.314	3.576 ± 3.157–3.8786	0	0
4.5		0.289 ± 0.254–0.306	3.483 ± 3.058–3.698	1.3 ± 1.144–1.378	15.674 ± 13.761–16.640
20		0.298 ± 0.268–0.311	3.605 ± 3.230–3.738	5.967 ± 5.359–6.228	72.09 ± 64.602–74.755
40		0.290 ± 0.272–0.312	3.483 ± 3.267–3.748	11.61 ± 10.885–12.492	139.32 ± 130.624–149.910
100		0.230 ± 0.205–0.252	2.758 ± 2.460–3.021	22.98 ± 20.499–25.176	275.75 ± 245.983–302.117
1000		0.102 ± 0.091–0.118	N/A	101.875 ± 91.498–117.509	N/A
0	Workers	0.157 ± 0.135–0.164	1.875 ± 1.607–1.961	0	0
4.5		0.169 ± 0.164–0.203	2.025 ± 1.844–2.432	0.75 ± 0.682–0.908	9.11 ± 8.296–10.945
20		0.190 ± 0.166–0.213	2.297 ± 2.016–2.553	3.733 ± 3.230–4.248	45.93 ± 40.323–51.067
40		0.158 ± 0.137–0.174	1.917 ± 1.676–2.093	6.302 ± 5.493–6.944	76.68 ± 67.054–83.721
100		0.161 ± 0.136–0.173	1.934 ± 1.725–2.092	16.054 ± 13.640–17.292	193.4 ± 172.506–209.158
1000		0.091 ± 0.076–0.100	N/A	91 ± 76.491–100.010	N/A

test, *bmct*) for all variables and were obtained by using the function mcompare (*bonferroni*). If sex differences were found, drones and workers were separated to facilitate analysis. Whenever appropriate, the means ± the standard error (SE) or medians and 95% Confidence Intervals (CI) are given in the text. All statistical figures were created using NCSS20 ("NCSS," 2020).

Depending on the analysis of residuals for the variable's consumption [g day^{-1}], exposure [ng day^{-1}], initial mass [g], mass difference [g], and sperm quantity [thousands], GLMs were modelled with Gaussian, Gamma or Poisson distribution using the function *glm*. Counter transforming the outcome variables, we opted for the gamma family that provided good fits (normality of the residuals). As sperm viability is a score ranging from 0 to 100%, a proportioned ordered logistic model was fit using the function *ologit*. Survival times for individuals were fitted using the function *mestreg* for multilevel survival models with a Weibull distribution. Survival was calculated by using cumulative survival rates [%] 12 days post experiment initiation. HPG length was analyzed with the *meglm* function where specimen was included as a random factor to account for the 30 repeated measured in each bee.

3. Results

3.1. Daily consumption and exposure

Control drone and worker daily consumption significantly differed (*bmct*; $p < 0.001$), with drones (0.293 ± 0.261–0.314) consuming about 46% more than workers (0.157 ± 0.135–0.164) (median ± 95%

CI [g day^{-1}]; Table 1). Subsequently, sex-specific differences were also observed among the remaining treatment groups (*regress*; $F_{(2, 378)} = 147.7$, *Adj.* $R^2 = 0.44$, $t = -15.7$, $p < 0.001$), where males consumed significantly more than the workers (*bmct*; all p's < 0.001). Drones from the 100 ng ml^{-1} (0.230 ± 0.205–0.252) and 1000 ng ml^{-1} (0.102 ± 0.091–0.118) treatment consumed significantly less than the remaining treatments (*bmct*; all p's < 0.0016; median ± 95%CI [g day^{-1}]); revealing a decrease in consumption by 22% and 65%, respectively. In contrast, a significant difference in consumption for workers was only observed in the 1000 ng ml^{-1} treatment (0.091 ± 0.076–0.100; median ± 95% CI [g day^{-1}]), resulting in a reduction of 42%. Based upon the consumption data, drones were exposed to significantly higher dosages of thiamethoxam compared to workers for each tested concentration (*bmct*; all p's < 0.001; Table 1). For both sexes, exposure significantly increased with increasing treatment concentration (*bmct*; all p's < 0.001). Detailed information on daily and total consumption as well as exposure values for both sexes and treatments can be found in Table 1.

3.2. Survival

Control drone and worker survival did not significantly differ (*bmct*; $p = 1.00$), with cumulative survival being above 93% for both sexes. Likewise, sex-specific differences across all treatment groups were not observed (*bmct*; $p = 0.561$, Fig. 2A & B). For both sexes, no significant differences for survival were found among controls and treatments up to 100 ng ml^{-1} (*bmct*; all p's $= 1.00$), with cumulative survival ranging between 93.4 and 100%. In contrast, the 1000 ng ml^{-1} treatment
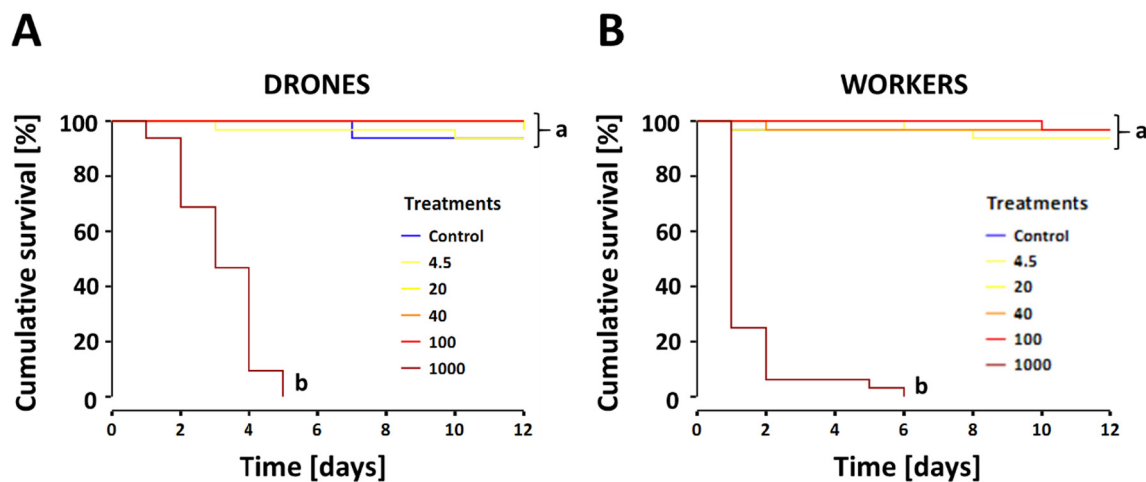
Fig. 2. Survival of *Bombus terrestris* drones and workers to multiple neonicotinoid insecticide (thiamethoxam) concentrations. Survival curves (Kaplan-Meier) indicate the cumulative survival [%] of (A) drones and (B) workers over the 12-day experiment for each treatment. No sex-specific differences were found (*mestreg*: $p = 0.561$). Different letters indicate a significant difference between treatments (*bmct*: all p's < 0.001).

showed significantly reduced survival (*bmct*; all *p*'s > 0.001), as all bees died within six days. Therefore, no individuals from the 1000 ng ml^{-1} treatment were considered for the following assessments.

3.3. Body mass

A significant difference was observed between drone and worker initial mass (*regress*; $F_{(2, 381)} = 132.5$, *Adj. R^2* = 0.41, $t = -16.3$, $p < 0.001$; Electronic Supplementary Materials Fig. S1), with drones (0.355 ± 0.04) weighing ~30% more than workers (0.255 ± 0.01; mean ± SE [g]). For both sexes, initial mass did not significantly differ among the treatments after the bees were randomly assigned to their respective cages (*bmct*; all *p*'s > 0.07). Body mass difference for both sexes was significantly positively correlated with exposure (*regress*; *Drones* $F_{(2, 381)} = 132.5$, *Adj. R^2* = 0.32, $p < 0.001$, drones $t = 6.93$: Workers $F_{(2, 150)} =$

19.34, *Adj. R^2* = 0.19, $t > 5.68$, $p < 0.001$; Fig. 3A, C) and negatively correlated with initial mass (*regress*; Drones $F_{(2, 381)} = 132.5$, *Adj. R^2* = 0.32, $t < -6.02$, $p < 0.001$; Workers $F_{(2, 150)} = 19.34$, *Adj. R^2* = 0.19, $t < -2.72$, $p < 0.001$). Control drones (0.004 ± 0.011) did not significantly differ in body mass from the 4.5 ng ml^{-1} treatment (0.036 ± 0.014) (*bmct*; $p > 0.85$, Fig. 3B; mean ± SE [g]). However, the 20, 40 and 100 ng ml^{-1} treatment drones gained significantly more mass when compared to the controls (*bmct*; all *p*'s < 0.04), despite revealing a reduced consumption. The mass difference was 0.063 ± 0.014, 0.094 ± 0.012 and 0.100 ± 0.013 (mean ± SE [g]), for the 20, 40, and 100 ng ml^{-1} treatments respectively, which represented an approximate 1500, 2300 and 2400% mass increase compared to the controls. In contrast, control workers (0.015 ± 0.008) only significantly differed from the 100 ng ml^{-1} treatment group (0.053 ± 0.008; mean ± SE [g]); reflecting an approximate increase in body mass by 250%
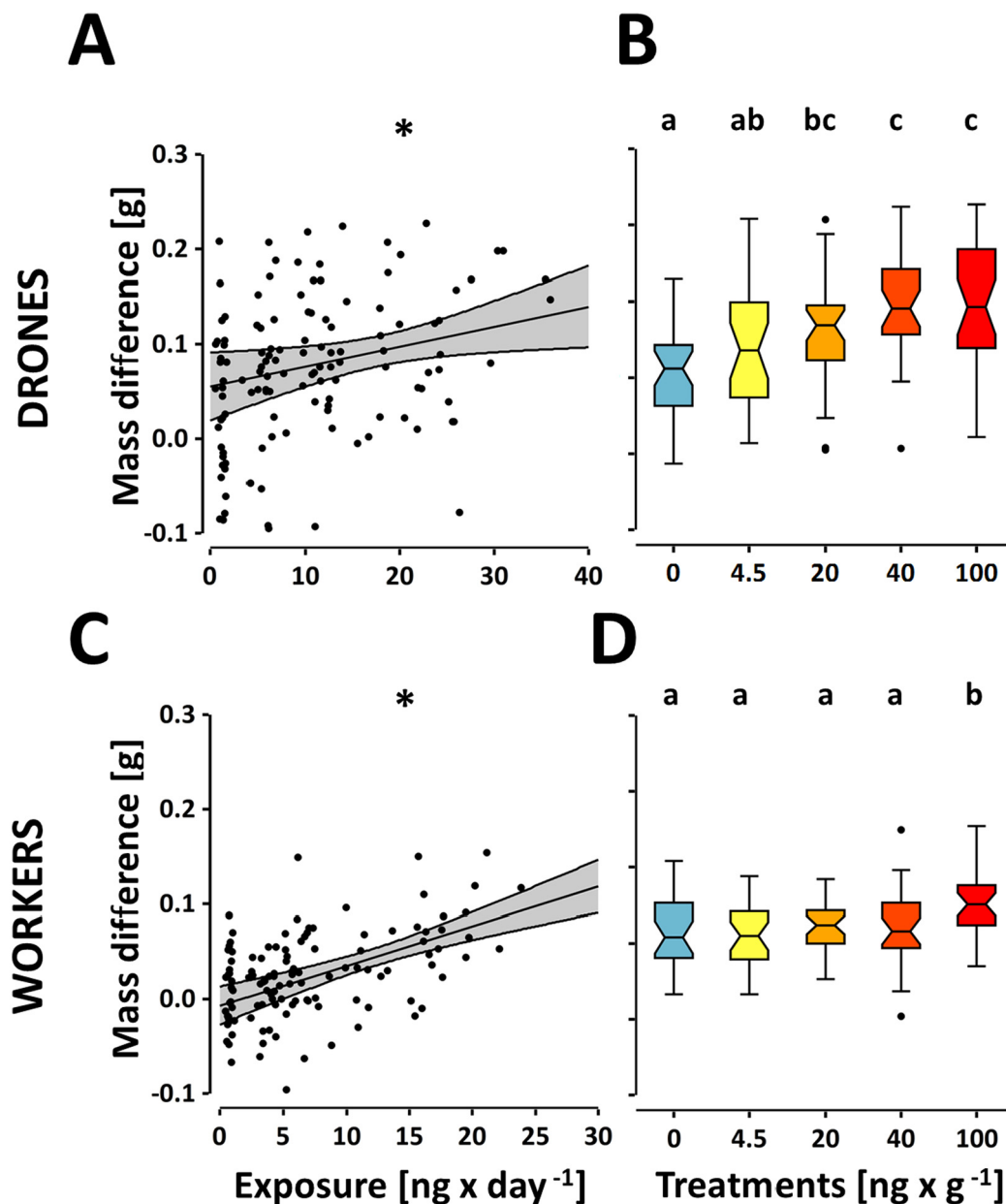

Fig. 3. Body mass difference of *Bombus terrestris* drones and workers exposed to various neonicotinoid insecticide (thiamethoxam) concentrations. Linear regression for body mass difference [g] and exposure [ng x day^{-1}] for (A) drones and (C) workers. A significant positive correlation for body mass difference was observed with increasing exposure for both sexes (* indicates $p < 0.001$). The boxplots show an increase in body mass gained as the treatment concentrations increase for drones (B), and similarly, an increase in mass gained in the 100 ng ml^{-1} treatment for workers (C). The boxplots show the inter-quartile range (box), the median (line within box), data range (vertical lines from box), and outliers (black dots). Different letters indicate a significant difference between treatments within each sex from post-hoc analysis of the GLMs (*bmct*: all *p*'s < 0.048).

compared to the controls. Furthermore, the workers from the 100 ng ml^{-1} treatment group significantly differed from all remaining treatment groups (*bmct*; all p's < 0.04; Fig. 3D).

3.4. Sperm assessments

Sperm quantity was not significantly correlated with initial mass or exposure (*regress*; $F_{(2,147)} = 0.99$, *Adj.* $R^2 < 0.001$, both p's > 0.19; Fig. 4A), and no significant differences were found between controls (390.5 ± 45.7) or among treatments (*bmct*; all p's > 0.5; Fig. 4B; mean ± SE [thousands]). On the other hand, sperm viability was significantly negatively correlated with exposure (*regress*: $F_{(2,145)} = 44.91$, *Adj.* $R^2 = 0.37$, $t < -8.86$, $p < 0.007$; Fig. 4C), but not with initial mass ($t = -1.83$, $p = 0.07$). Controls revealed the highest sperm viability (86.6 ± 0.79) and significantly differed from the remaining treatment groups (*bmct*; all p's < 0.02; Fig. 4D; mean ± SE [%]). No significant difference was observed between 4.5 and 20 ng ml^{-1} (*bmct*; $p = 0.882$) or between the 20 and 40 ng ml^{-1} treatment groups (*bmct*; $p = 1.00$); however, all treatment groups significantly differed from the 100 ng ml^{-1} treatment (62.3 ± 2.31; mean ± SE [%]). In comparison to the controls, this resulted in a reduction in sperm viability for the 4.5, 20, 40 and 100 ng ml^{-1} treatments by 10, 15, 18, and 28%, respectively.

3.5. Hypopharyngeal glands

A significant positive correlation between HPG size and initial mass was found (*regress*; $F_{(2, 2818)} = 203.6$, *Adj.* $R^2 = 0.14$, $t = 13.5$, $p < 0.001$), whereas a significant negative correlation with exposure was revealed (*regress*; $F_{(2, 2818)} = 203.6$, *Adj.* $R^2 = 0.14$, $t = -15.3$, $p < 0.001$; Fig. 4E). Controls had the largest acini (49.6 ± 0.23) and significantly differed from all treatment groups (*bmct*; all p's < 0.008; Fig. 4F; mean ± SE [μm]). No significant difference was observed between 4.5 and 20 ng ml^{-1} (*bmct*: $p = 1.00$); however, all treatment groups significantly differed from the 40 (45.2 ± 0.22) and 100 ng ml^{-1} (44.5 ± 0.24) treatments, which had the smallest acini (*bmct*; all p's < 0.008; mean ± SE [μm]). In comparison to controls, this resulted in a reduction of HPG acini width by 5, 5, 9 and 10% for the 4.5, 20, 40 and 100 ng ml^{-1} treatments, respectively.

4. Discussion

The data reveal that chronic exposure to the neonicotinoid thiamethoxam can negatively impact important physiological traits of drones and workers, relevant for bumblebee colony fitness. While field-realistic exposures (≤11.61 ng day^{-1}) of the neonicotinoid did not affect survival, they did have striking effects on hypopharyngeal gland (HPG) acini width and sperm viability, even at the lowest exposures (0.75 and 1.3 ng day^{-1}, respectively), similar to honeybees (Renzi et al., 2016; Straub et al., 2016). This suggests a trade-off between survival and fitness components, possibly due to costly detoxification (Castañeda et al., 2009). In light of ubiquitous use of neonicotinoids in the environment, and the key roles of functional drones and workers, the results indicate mechanisms for reduced bumblebee colony fitness and population declines (Arce et al., 2017; Whitehorn et al., 2012).

Chronic exposure to field-realistic concentrations (i.e., below 40 ng ml^{-1}) revealed no significant effect on sucrose consumption. Therefore, caloric restriction and starvation appear unlikely to compromise detoxification (Turturro et al., 2000). In contrast, high concentrations (i.e., 100 and 1000 ng ml^{-1}) significantly reduced consumption, which supports earlier studies (Laycock et al., 2014). This may be attributed to learned avoidance or to neurotoxins reducing the ability or willingness to feed (Laycock et al., 2014; Muth et al., 2020; Williamson et al., 2014). Drones from all treatments consumed significantly more than their worker counterparts, possibly due to variation in body size and/or metabolism (Heinrich, 1972). These results should be interpreted with caution, as energy requirements may differ substantially in the field. Furthermore,

proteins were not supplied, which may have had an impact on sucrose consumption as well as on detoxification (Alaux et al., 2010). In any case, drones were exposed to higher neonicotinoid dosages than workers, highlighting the urgent need to incorporate males in ecotoxicological studies.

High survival rates (>93%) were found for both drones and workers in the controls, as well as in the treatments up to 100 ng ml^{-1}, which supports previous findings (Hopwood et al., 2016; Mommaerts et al., 2010). However, as bumblebee workers may live for several months, mortality due to neonicotinoid exposure may have been revealed if assessments were performed for a longer duration, and bumblebees needed to perform necessary colony tasks (i.e., foraging or brood care) (Goulson, 2003). Nevertheless, there are numerous studies showing clear negative effects of agrochemicals on bumblebee fitness, yet not directly on survival (Arce et al., 2017; Baron et al., 2017a; Dance et al., 2017; Elston et al., 2013; Laycock et al., 2014; Whitehorn et al., 2012; Wu-Smart and Spivak, 2018). This may be due to a trade-off between survival and fitness (Harshman and Zera, 2007; Schwenke et al., 2016), as insect detoxification is evidently costly (Castañeda et al., 2009; du Rand et al., 2015). The necessary allocation of resources to ensure survival via detoxification may come at the expense of other physiological functions (Hosken, 2001; Sheldon et al., 1996; Siva-Jothy et al., 1998). Indeed, a focus on survival and consumption only would have masked the negative impacts revealed on physiology.

Indications of impaired physiology became apparent as drones exposed to concentrations above 20 ng ml^{-1} and workers at 100 ng ml^{-1} significantly gained body mass compared to controls. This may be attributed to reduced activity levels of chronically exposed bees (Cresswell et al., 2014; Gill and Raine, 2014; Tosi et al., 2017), a metabolic dysregulation, or the downregulation of genes involved in sugarmetabolism and glycolytic pathways (Christen et al., 2018). Regardless of the underlying mechanism, altered body mass and impaired energy metabolism may reduce flight capacity (Kenna et al., 2019; Tosi et al., 2017) and thermogenesis (Heinrich, 1972; Schultze-Motel, 1991), which can reduce bumblebee colony fitness (Kraus et al., 2009; Mommaerts et al., 2010; Darveau et al., 2014). Clearly, field studies and molecular data are required to confirm these laboratory findings.

Collaborative brood care is a fundamental component of eusociality and clearly depends on optimal worker performance. The data clearly show for the first time that chronic thiamethoxam exposure can significantly reduce HPG acini width in bumblebees, which may impair their ability to attend brood. The smaller control acini width (~50 μm) compared to earlier data (~60 μm, Albert et al., 2014) may be due to the experimental bees being deprived of pollen (see Brodschneider and Crailsheim, 2010) and not attending brood (Lass and Crailsheim, 1996). Nonetheless, thiamethoxam significantly decreased HPG acini width even at the lowest tested exposure (0.75 ng day^{-1}). These results are congruent with prior studies on honeybees (Hatjina et al., 2013; Renzi et al., 2016), and are likely due to disruptions in cellular development (Jedlicka et al., 2016; Pereboom, 2000; Wessler et al., 2016). It remains to be tested if this physiological response is linked to a behavioral effect. Reduced HPG acini width may help explain reduced nursing behavior and early shifts from nursing to foraging in both neonicotinoid-exposed bumblebees, *B. impatiens* (Crall et al., 2018) and honeybees (Hatjina et al., 2013). Given that sublethal effects on HPGs negatively affect collaborative brood care, this will have drastic consequences for colony development and the production of sexuals.

As spermatogenesis and spermiogenesis are completed upon adult emergence in all bee species (Hoage and Kessel, 1968), it seems plausible that neonicotinoid exposure during adulthood does not affect sperm quantity. Indeed, while neonicotinoid exposure had no significant effect on sperm quantity, sperm viability was negatively affected even at the lowest exposure (1.3 ng day^{-1}). This is of concern as it is crucial that the transferred semen is viable (Ciereszko et al., 2017). Reduced sperm viability may be due to increased oxidative stress (Ciereszko et al., 2017), impaired function of mitochondria (Baer et al., 2009), or
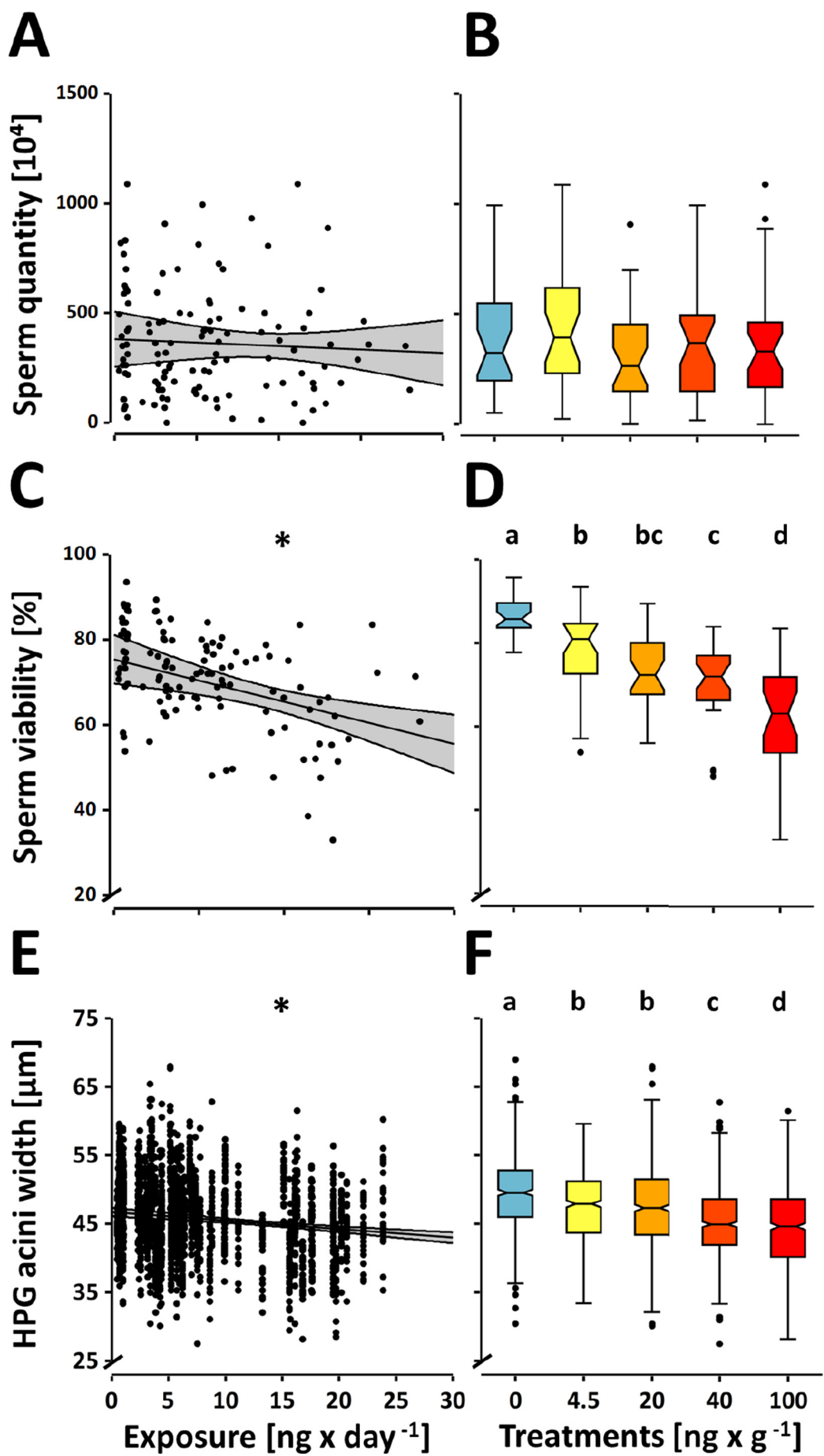
reduced seminal fluid protein abundance (Baer et al., 2009), yet these mechanisms remain to be tested in bumblebees. While similar effects have been reported in polyandrous honeybees (Straub et al., 2016), honeybee workers can replace insufficiently mated queens via rearing a new one, whereas, bumblebees lack this opportunity. Moreover, polyandry may buffer the effects of mating with males possessing poor sperm viability. Thus, the consequences for single mated species, ranging from solitary bees over most bumblebees to ants, are likely to be far more severe, pending the impact on sperm viability and life history of the species. Our findings may further explain the observed long-term effects of neonicotinoids on ant colony development (Schläppi et al., 2020). While the amount of sperm transferred during copulation has not been shown to be a limiting factor in bumblebees (Baer and Schmid-Hempel, 2000), the effect of sperm viability on fitness is yet to be explored. As eggs must be fertilized in almost all species, impaired sperm traits may result in non-hatching eggs or a male-biased sex-ratio depending on sex-determining mechanism of the species in question. Ultimately, factors affecting reproductive success are likely to influence not only individual fitness, but also the dynamics of entire populations (Lumley et al., 2015). Thus, our observations on reduced male quality may help understand recent observations of reduced insect populations (Wagner, 2020).

While often subject to much criticism (Carreck and Ratnieks, 2014), yet indispensable for ecotoxicology, laboratory studies enable controlled environments that facilitate more precise measurement of chemical concentrations and their effects (Neumann et al., 2015). As our laboratory conditions most likely reflect ideal circumstances (i.e., constant temperature and humidity; *ad libitum* sucrose-solution; no need to perform colony relevant tasks, such as brood attendance or foraging), the findings are therefore likely conservative when compared to the field, especially considering that neonicotinoids have been shown to cause negative effects on bees when combined with other stressors (Sanchez-Bayo and Goka, 2014; Straub et al., 2019). Subsequently, colony-level field studies are most appropriate for ecotoxicological purposes (Arce et al., 2017; van Oystaeyen et al., 2020); yet may not always be feasible due to difficulties with standardizing environmental conditions (Neumann et al., 2015). Furthermore, the endpoint of individual bee survival is still widely used, which may lead to inappropriate conclusions and policy decisions. If future risk assessments still focus on individuals, it seems imperative to measure fitness components to adequately evaluate the hazards of xenobiotics. Ultimately, exclusively focusing on measures of fitness in the field or laboratory setting should be the goal (Straub et al., 2020) if our aim is to protect natural biodiversity and mitigate the evident role of agrochemicals for the ongoing mass extinction of species.

In conclusion, despite not measuring colony fitness directly, the data provide evidence that neonicotinoids can adversely impact fitness components, thereby providing additional mechanistic explanations for recent observations in bumblebee declines. Although trade-offs between survival and reproduction are well documented, such mechanisms remain widely overlooked in insect ecotoxicology. Indeed, the data support that negative evidence for effects on survival are likely misleading in ecotoxicology, as essential physiological endpoints appear far more vulnerable at field-realistic levels. Furthermore, we urge that future ecotoxicological risk assessments incorporate both sex and caste (i.e., queens, drones, and workers) due to the observed sex-specific consumption resulting in varying exposure scenarios and the key role of sexuals for population dynamics. Ultimately, to prevent further irreparable damage to our ecosystems, action must be taken to overhaul the prophylactic usage of pesticides in agricultural systems and policymakers should reinforce the implementation of integrated pest management strategies, where pesticides only act as a last resort (Brühl and Zaller, 2019; Wyckhuys et al., 2021).

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CRediT authorship contribution statement

Angela Minnameyer: Formal analysis, Investigation, Writing – original draft, Visualization. **Verena Strobl:** Investigation. **Selina Bruckner:** Investigation. **Domenic W. Camenzind:** Investigation. **Annette Van Oystaeyen:** Resources, Writing – review & editing. **Felix Wäckers:** Resources, Writing – review & editing. **Geoffrey R. Williams:** Writing – review & editing, Resources, Supervision, Funding acquisition. **Orlando Yañez:** Investigation. **Peter Neumann:** Writing – original draft, Resources, Supervision, Funding acquisition. **Lars Straub:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Project administration, Funding acquisition.

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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Fig. 4. Sperm traits and hypopharyngeal (HPG) acini width analysis of *Bombus terrestris* exposed to various neonicotinoid insecticide (thiamethoxam) concentrations. (A, B) Sperm quantity [thousands] (C, D) sperm viability [%] for drones and (E, F) HPG acini length for workers at day 12. Linear regression was used to investigate the correlations between response variables and exposure [ng x day^{-1}], where * indicate $p < 0.001$. The boxplots show the inter-quartile range (box), the median (line within box), data range (horizontal lines from box), and outliers (black dots). Different letters indicate significant difference among treatments (*bmct*: all *p*'s < 0.008).

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