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Negative effects of neonicotinoids on male honeybee survival, behaviour and physiology in the field

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Abstract

1. Agricultural chemicals such as neonicotinoid insecticides are believed to be one important factor responsible for the recent reduction in health of pollinating insects like the western honeybee *Apis mellifera*. However, effects of neonicotinoids on male (drone) honeybee health remain severely understudied.
2. We examined for the first time the multidimensional effects of field-realistic concentrations of two common neonicotinoid insecticides (thiamethoxam and clothianidin) on drone honeybee survival, behaviour and physiology using individuals reared and maintained as adults in the field.
3. Our data showed that neonicotinoids reduced honeybee drone survival by 51%, increased drifting behaviour to non-maternal colonies by 100%, delayed flight activities by 3 days and reduced number of living sperm by 28%. However, they did not influence the sperm concentration produced by the drones, the strength of the drone's maternal colonies or the total number of drones produced by those colonies.
4. *Policy implications*. Our study demonstrated that neonicotinoids can elicit a diverse array of lethal (survival) and sublethal (behaviour, reproductive physiology) effects on male honeybees *Apis mellifera* in the field. These findings should be considered by policy makers looking to adopt and implement science-based, holistic risk assessments to more comprehensively assess effects of chemicals on important ecosystem service providing insects like the honeybee. To date, risk assessment schemes do not specifically address potential effects on male bees.

KEYWORDS

agro-chemical, *Apis mellifera*, drifting, honeybee, neonicotinoid, reproductive trait, sperm, thiamethoxam

1 | INTRODUCTION

Understanding and properly managing the influence of anthropogenic effects on insects are critical to ensuring the delivery of many essential ecosystem services (Elmqvist et al., 2003; Sánchez-Bayo & Wyckhuys, 2019). When examining the influence of stressors on insects, researchers are frequently challenged to perform scientifically sound investigations that reflect real-world conditions. Most often, established protocols are confined to laboratory studies of limited breadth that focus on lethal consequences of stressors or physiological responses that may be strongly influenced by the unnatural in vitro conditions in which they are performed (Ghallab & Bolt, 2014; Hartung & Daston, 2009; Retschnig et al., 2015; Williams et al., 2013). Although these laboratory-based studies have many benefits that allow for data comparison, like controlled environmental conditions and high-precision exposure scenarios, it can be challenging to extrapolate those data to real-world scenarios that allow for the understanding of the mechanisms responsible for recent observations of population declines in many bee species, as well as increased managed honeybee colony mortality (Goulson et al., 2015; Gray et al., 2020; Kulhanek et al., 2017).

Bees are among the most well studied of insects, yet knowledge concerning their health and well-being is also disproportionally generated under laboratory conditions. Prized for their contribution to the maintenance of plant biodiversity and agricultural crop productivity, bees are often used as an indicator of environmental condition. Alteration to habitat, severe weather events and climate change, invasive species and environmental contamination are all believed to play important roles in describing recent declines in unmanaged bee species and increased mortality of managed ones (Goulson et al., 2008; Neumann & Carreck, 2010; Potts et al., 2010; Powney et al., 2019; Williams et al., 2010).

Neonicotinoid insecticides have recently received considerable attention as a possible important stress factor to bees (Müller, 2018). Due to their broad-spectrum activity, as well as high efficiency, they are among the most commonly applied insecticides worldwide (Elbert et al., 2008; Jeschke et al., 2011). Studies have shown that they can cause significant lethal and sublethal effects in multiple bee species, for example, by negatively affecting individual development and behaviour (Friedli et al., 2020; Schneider et al., 2012; Tavares et al., 2019). The eusocial western honeybee *Apis mellifera* has historically served as a model organism for ecotoxicology studies, especially for the neonicotinoids. This is mainly because of their high economic value, because their biology is well known and because they can be easily reared and maintained (EFSA, 2014). Similar to other taxa, most studies are performed under laboratory conditions because of financial constraints, ease of observation and control of environmental conditions (Carreck & Ratnieks, 2014; Henry et al., 2015; Neumann et al., 2015; Rortais et al., 2017; Sanchez-Bayo, 2014). These studies facilitate the interpretation of results by controlling as many environmental variables as possible (Calisi & Bentley, 2009) and have proved useful for anticipating acute effects in the field (Fischer & Moriarty, 2014). However, unlike field studies, they can also be limited in scope because they may lack the potential to investigate real-world scenarios involving organisms living in their natural environment. Conversely, field studies are more likely to be influenced by environmental factors that can lead to difficulty in standardizing experiments and interpreting results (Cutler et al., 2014; Rortais et al., 2017; Simon-Delso et al., 2017; Woodcock et al., 2016).

The majority of honeybee studies have focused on females, specifically the primarily non-reproductives called workers, whereas males, known as drones, and the primary reproductive females, known as queens, are often overlooked. This is especially worrisome given that poorly inseminated queens are prone to be killed and replaced, at a cost, by the colony, and because recent studies suggest that poor queen health is at least partially responsible for recent increases in honeybee colony mortality (Amiri et al., 2017; Brodschneider et al., 2018a; Genersch et al., 2010; Kulhanek et al., 2017; vanEngelsdorp & Meixner, 2010). Recent studies also suggest that honeybee drones, which are haploid like most male hymenopterans (de la Filia et al., 2015; Normark, 2002), might be more susceptible to neonicotinoids and other stressors compared to their female diploid counterparts because of hemizygosity at immune and detoxification loci (Friedli et al., 2020; O'Donnell & Beshers, 2004).

Studies that have examined the effects of neonicotinoid insecticides on honeybee reproductives are relatively rare. Those that exist have generally observed broad negative effects on queen fecundity, physiology and behaviour (Brandt et al., 2017; Chaimanee et al., 2016; Sandrock et al., 2014; Williams et al., 2015; Wu-Smart & Spivak, 2018). It is possible that these effects can translate to a reduction in the number of successful matings (Forfert et al., 2017), despite not influencing the timing and duration of mating flights (Williams et al., 2015); similar negative effects on survival and physiology have been seen in drones (Abdelkader et al., 2019; Ciereszko et al., 2017; Grassl et al., 2018; Straub et al., 2016b). For example, thiamethoxam reduced drone survival in both the laboratory and the field (Grassl et al., 2018; Straub et al., 2016a), whereas clothianidin exposure lead to decreased protein content in semen, which might reduce sperm quality (Abdelkader et al., 2019). To date, few data exist on how neonicotinoids may affect traits important to honeybee drone reproduction, like physiological ones such as sperm quantity and viability, or behavioural ones such as orientation to and from mating sites (Orr & Garland, 2017). This is primarily because laboratory studies cannot provide a suitable testing ground to properly examine the many functions, processes and behaviours that are so critical to drones fulfilling their primary roles as reproductives.

Here, we examined the effects of neonicotinoid insecticides on honeybee drones in the field. We accomplished this by using an established method that employed free-flying adult *A. mellifera* honeybee drones that were exposed to chronic field-realistic concentrations of two commonly applied neonicotinoids—thiamethoxam and its primary metabolite clothianidin. Based on previous studies that revealed that honeybee workers exposed to neonicotinoid insecticides exhibited significantly reduced orientation abilities (Fischer et al., 2014; Tison et al., 2016) and that drones showed increased mortality and reduced sperm capacities during a complementary

laboratory study (Straub et al., 2016b), we hypothesized that drones exposed to neonicotinoids would experience both significant lethal (survival) and sublethal (behavioural and physiological) effects.

2 | MATERIALS AND METHODS

2.1 | Study site setup

The study was performed in Bern, Switzerland, between April and September 2016 at the Behavioural Ecology Research Station of the University of Bern, on the outskirts of the city where there is a low density of beekeeping. The surrounding area consisted of mixed land-use types, but was primarily composed a large deciduous forest managed for recreational purposes, followed by mixed agricultural areas of crop and pasture lands. Some medium-density residences and a large river were also within flight range of colonies. Twenty local *A. mellifera* honeybee colonies were established in the same location in early May using the shook swarm method (Delaplane et al., 2013). Each colony was headed by a laying sister queen, which had mated locally, 2.0 kg workers and six Dadant frames equipped with organic worker cell wax foundation that was previously examined for agricultural chemical residues by the University of Hohenheim (see Figure S1). Colonies were provided with 5 L of 1:1 (w:w) sucrose solution composed of white granulated sugar and tap water, as per standard beekeeping practices, to encourage colony growth and to reduce the need for colonies to search out nectar sources during the study (Free, 1965).

2.2 | Insecticide exposure

Similar to previous studies performed over multiple years (Forfert et al., 2017; Straub et al., 2016b, 2019; Williams et al., 2015), experimental colonies were exposed to treatments via in-hive feeding of pollen patties. This allows us to make comparisons among studies and to generate hypotheses for future experiments. Each colony was afforded 21 days to establish, before being randomly assigned to one of two treatment groups (neonicotinoid or control) and fed daily by placing 100 g pollen paste composed of 60% fresh corbicular pollen, 30% sugar powder and 10% organic honey on top of the broodnest within the hive (Sandrock et al., 2014; Straub et al., 2016b; Williams et al., 2015). Honeybee collected corbicular pollen and honey was obtained from Bienen Roth® (Wila) and MieleBio (Vezio) respectively. The corbicular pollen was tested for 42 common industrial agrochemicals, including thiamethoxam and clothianidin, by the French National Centre for Scientific Research using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Pollen patties provided to the neonicotinoid colonies additionally contained 4.5 ppb thiamethoxam and 1.5 ppb clothianidin (both Sigma-Aldrich), which reflected field-realistic concentrations observed in honeybee collected pollen from multiple sources, including thiamethoxam- or clothianidin-treated maize (Pilling et al., 2013) and squash (Stoner & Eitzer, 2012), as well as in herbaceous plants (Wood et al., 2019) and wild flowers (Botias et al., 2015). Similar concentrations have also been detected in pollen stored within the colony (Bonmatin et al., 2015; David et al., 2016; Mogren & Lundgren, 2016; Tong et al., 2018), which is also called beebread (Crailsheim, 1990). This exposure corresponded to similarly themed studies using these agricultural chemicals as models (Forfert et al., 2017; Straub et al., 2016b; Williams et al., 2015). Addition of thiamethoxam and clothianidin to the neonicotinoid treatment pollen patties occurred during patty formation. In brief, pure analytical standards of both neonicotinoids were used (with purities of >99%; Sigma-Aldrich®) and dissolved in distilled water (1 mg/L). Aliquots of a single stock solution for each compound were added to the honey, which was then thoroughly mixed by kneading the components of the patties in a large plastic container (63 L) until a homogenous paste was made. Concentrations were confirmed by the French National Centre for Scientific Research using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Similar to previous studies (Friedli et al., 2020; Grassl et al., 2018; Straub et al., 2016b; Williams et al., 2015), colonies were then fed pollen patties for 50 days to ensure that they were exposed for at least two complete brood cycles (Winston, 1991). Previous studies suggest that foraging honeybees can be exposed to neonicotinoid residues for a similar period of time due to overlapping treated crop blooming periods (Tsvetkov et al., 2017), contaminated planter dust that is exhausted to the environment during and after planting (Krupke et al., 2012) and crops and neighbouring non-agricultural foraging areas being contaminated as a result of water run-off (Botias et al., 2015; Long & Krupke, 2016; Mogren & Lundgren, 2016; Schaafsma et al., 2015). Throughout the entire exposure period, each colony was equipped with an entrance pollen trap to promote feeding on the experimental pollen patties (Sandrock et al., 2014).

2.3 | Colony strength

Twenty colonies were assessed using the Liebefeld estimation method that visually quantified bees, capped and uncapped brood, honey and pollen in each colony (Delaplane et al., 2013; Gerig & Imdorf, 1983). Assessments were carried out immediately before initial pollen patty treatment exposure (day 0) and 10 days post-exposure (day 60). Colony strength variables [i.e. total bees, total capped brood surface (cm^2), total uncapped brood surface (cm^2), total honey surface (cm^2) and total pollen surface (cm^2)] were first evaluated as percent scores ranging from 0 to 100 of frame coverage and then converted into absolute values of area (cm^2) [e.g. total capped brood surface (cm^2)] or weight (kg) [e.g. honey (kg)] following Delaplane et al. (2013).

2.4 | Source of drones

To promote drone production for the experiment, one frame containing drone comb foundation was provided to each colony 21

days after initial experimental treatment exposure. Seventeen days later, when drone frames were suitably prepared (i.e. wax cells produced by workers), queens from each colony were caged for 48 hr to the drone frame to obtain sufficient quantities of drones of a similar known age cohort. These drone brood frames remained within their respective colonies for the entire duration of drone development—roughly 24 days (Winston, 1991). Drone frames remained caged for an additional 24 hr post expected natural emergence time to allow drones to emerge naturally within their hives.

2.5 | Drone quantity, survival and drifting behaviour

Twenty-four hours post natural emergence, individual drones were removed from the frame cages, coloured with colony-specific non-toxic acrylic paint and then placed back into their respective maternal colonies unrestricted so that they could move freely and perform orientation and mating flights naturally. A number of drones produced (range: 18–509 individuals per colony) were quantified by counting the total number of marked individuals from each colony. To assess daily survival and drifting behaviour, colonies were visually inspected every day until 99% of all neonicotinoid drones (18 drones) were no longer observed. Drone survival rates were assessed by comparing the daily total marked drones present in each colony for each colour, regardless of which colony there were in, to the total number of marked individuals in each colony on the first day of the experiment. Drifting, defined as the movement of individuals from their maternal colony to a non-maternal one (Butler, 1939), is attributed to the consequences of orientation errors during orientation or mating flights (Free, 1956; Neumann et al., 2000; Pfeiffer & Crailsheim, 1998; Rauschmayer, 1928), and acceptance by non-maternal guard bees (Moritz & Neumann, 2004). It was assessed for our drones daily by comparing the presence of colony-specific coloured drones in their maternal versus non-maternal experimental colonies. Therefore, we obtained an individual percentage of drifted drones per colony for each day. Daily mortality and drifting occurrence assessments were carried out early each morning between 7.00 and 9.00 a.m., prior to any orientation or mating flights later in the day (Currie, 1987; Johnson et al., 2013; Reyes et al., 2019).

2.6 | Sperm assessment

Fourteen days post natural experimental drone emergence (day 14), a subset of 20–30 individuals per colony was sampled to assess sperm traits at the typical age of drone maturation (Rhodes et al., 2011; Woyke & Ruttner, 1958). These individuals were censored from the drone survival analysis (Wei, 1992). All drones from a given colony were placed in a separate metal hoarding cage (Williams et al., 2013) and immediately transported to the laboratory. Each drone was then carefully removed from its cage using featherweight forceps and dissected alive by pinning it to a wax plate (Mazeed & Mohanny, 2010). The entire reproductive tract, including the testis, mucus gland and seminal vesicle, was removed and placed in a 1.5-ml Eppendorf® tube containing 500 μl $Kiev^+$ buffer (Carreck et al., 2013). It was then crushed using an Eppendorf® micro-pestle and gently vortexed to create a homogenized stock sperm solution that was immediately used to assess sperm viability (proportion of sperm alive to dead; Hunter & Birkhead, 2002) and quantity.

In brief for sperm viability, a 50-μl aliquot from the stock sperm solution was inserted into a 1.5-ml Eppendorf® tube as described by Collins and Donoghue (1999). Each sample was diluted with 50 μl $Kiev^+$ buffer before stained with 2 μl of propidium iodide (PI) solution (1 mg/ml) and 1 μl of Hoechst 33,342 (0.5 mg/ml; both Sigma-Aldrich; Wegener et al., 2012). After 20 min of incubation at RT and complete darkness, tubes were again gently vortexed and 10 microlitres were mounted on a 24 × 24 mm coverslip so that it could be examined at 400× magnification using fluorescent microscopy (Olympus BX41) equipped with filter cubes for UV excitation (Wegener et al., 2012). Ten visual fields from each sample were evaluated to quantify the total living and dead sperm; an average value was used in subsequent analyses (Wegener et al., 2012). Sperm concentration was assessed for each individual by diluting 20 μl of respective sperm stock solution using 80 μl $Kiev^+$ buffer (1:5 dilution) in a 1.5-ml Eppendorf and gently vortexed. Ten microlitres from each sample was mounted on a Neubauer haemocytometer (depth 0.1 mm, 1/400 mm^2) and examined under light microscopy (Thermo Fisher Scientific). Sperm concentration for each individual was assessed according to Rhodes et al. (2011) using the following formula: Sperm concentration = total number of sperm cells in five large haemocytometer squares x dilution factor (1:5) × 50,000. Using both the sperm concentration and sperm viability values, the living sperm quantity was calculated by multiplying the two factors together.

2.7 | Statistics

Statistical analyses were performed using STATA16 (StataCorp, 2019), whereas statistical figures were created using NCSS v.12 (NCSS 2016, 2018). All outcome variables at the colony level (i.e. colony assessment variables and quantity of drones produced) and individual drone level [i.e. cumulative survival (% day^{-1}), drifting occurrence (% day^{-1}), sperm concentration (millions), sperm viability (%) and living sperm quantity (millions)] were tested for normality by using the Shapiro–Wilk's test and homogeneity of variances with the Levene's test and subsequent statistical methods were chosen accordingly (see Table S1).

Where colony-level outcome variables were normally distributed (Shapiro–Wilk's test, $p > 0.05$), treatment groups were compared using a one-way ANOVA and the STATA function '*ANOVA*'. If normality was rejected, groups were compared using a Kruskal–Wallis ANOVA (Shapiro–Wilk's test, $p < 0.05$) and the function '*kwallis*'. For outcome variables at the individual drone level [i.e. cumulative survival (% day^{-1}), drifting occurrence (% day^{-1}), sperm concentration

(millions), sperm viability (%) and living sperm quantity (millions)], two-level generalized regression mixed models with random intercepts were fitted, whereby treatment (neonicotinoid vs. control) was included as the fixed effect and the colony identification number as the random effect (Leckie, 2010). In these cases, individual drones were the units of analysis. Whenever possible, each two-level model was compared with its single-level counterpart using a likelihood ratio (LR) test (Sribney & StataCorp, 2005). All colony-level variables [apart from total capped brood surface (cm^2; Shapiro–Wilk's test, $p < 0.05$)], as well as drones produced, were normally distributed (Shapiro–Wilk's test, $p > 0.10$).

Survival time was set using the function *stset* and the *if* option was used for censored individuals. Differences in survival of drones between treatments were fitted using the '*mestreg*' function for multilevel survival models considering colony as the co-variate (Leckie, 2010) and data were plotted using Kaplan–Meier curves to visualize survival. Median longevity was calculated as the 50th percentile of survival time (Lee & Wang, 2003). Drones sampled on day 14 for sperm assessments were right censored. The incorporated right censoring, whereby the survival time of the drones is 'incomplete' at the right side of the follow-up period because they were killed for sperm assessments, is the most common type of censoring in survival analyses (Wei, 1992). The daily percentage of drifted drones [drifting occurrence (% day^{-1})] was non-normally distributed (Shapiro–Wilk's test for normality, $p < 0.001$), and presented a zero inflation effect. We therefore applied the STATA function '*zip*', which fits a zero-inflated Poisson model to count data with excess zero counts (Desmarais & Harden, 2013), taking both treatment group and time as fixed effects. This model properly captured excess zeros in drifting occurrence (% day^{-1}) by calculating regression coefficients separately for the zero inflation in both the treatment and time effects. The zero inflation was significant ($p < 0.001$ and 0.088) for time and treatment, respectively. As the main output, the model renders incidence rate ratios for treatment and time without the excess zeros. Sperm concentration and living sperm quantity at the individual drone level were both non-normally distributed (Shapiro-Wilk's test, $p < 0.001$), and were over-dispersed. Therefore, they were fitted to two-level negative binomial regression models using the '*menbreg*' STATA function (with the colony as a co-variate). Sperm viability was considered as a score ranging from 0% to 100% and was also non-parametric (Shapiro–Wilk's test for normality, $p < 0.001$). As a result, a two-level ordered logistic regression model was applied using STATA function '*meologit*' (Greene, 2012).

Whenever appropriate, either the arithmetic means ± the standard error (*SE*) or medians ± 95% confidence intervals (CI) of non-transformed values are given in the text. Median differences and their 95% CI were calculated for sperm concentration, sperm viability and living sperm quantity using the STATA16 package '*somersd*'. Lastly, percentage difference between treatment groups was calculated by dividing the difference between neonicotinoid and control variable values (i.e. mean or median) by the control variable value (i.e. mean or median), and then multiplied by 100.

3 | RESULTS

3.1 | Colony strength

Colony strength assessments revealed no significant difference for any of the evaluated parameters between control and neonicotinoid insecticide treatments (all *p*-values >0.25; see Tables S1 and S2).

3.2 | Drone quantity, survival and drifting behaviour

Quantity of drones produced did not significantly differ between treatment groups ($p = 0.94$), with 236.25 ± 60.08 and 229.67 ± 47.35 drones produced by control and neonicotinoid colonies respectively (mean ± *SE*; see Table S2). Median longevity of neonicotinoid drones (11 ± 10–11 days) was significantly lower than controls (16 ± 15–16 days; $p = 0.046$; median ± 95% CI, Figure 1). Additionally, survival 14 days post-emergence, when drones have typically matured (Rhodes et al., 2011), was significantly reduced for insecticides compared to controls; survival was 27.4 ± 25.1%–29.80% and 55.5 ± 53.3%–55.7% for neonicotinoids and controls respectively (cumulative survival ±95% day^{-1} CI; see Table S3). This corresponded to an ~51% increase in neonicotinoid drone mortality. Furthermore, a significant difference in the occurrence of drifting was observed between treatment groups ($p < 0.001$, Figure 2). For the first 5 days post-emergence, no drifting was observed for either treatment group; however, drifting was first noted at day 6 by control drones and day 9 by neonicotinoid drones (Figure 2, see Table S3). Daily mean drifting occurrence post-emergence across the examined 20 days was 31.24 ± 5.14% and 15.26 ± 3.63% for the neonicotinoid and control drones respectively (mean ± *SE*%; see Tables S2 and S3). This represented a 104% increase in drifting occurrence by the neonicotinoid treatment group when compared to the controls.

3.3 | Sperm assessment

No significant difference was observed between neonicotinoid insecticide and control drone sperm concentration ($p = 0.397$; Figure 3a), with 4.10 ± 3.67–4.65 and 4.22 ± 3.45–4.90 million sperm respectively (median ± 95% CI). However, sperm viability was significantly different between treatments ($p = 0.001$, Figure 3b). Neonicotinoid drones possessed 20.6% lower sperm viability (70.29 ± 68.15%–71.86%) than controls (88.58 ± 87.12%–91.18%; median ± 95% CI, see Table S2). Furthermore, living sperm quantity was significantly different between the two treatment groups ($p < 0.031$; Figure 3c), with neonicotinoid drones possessing 28% less living sperm than controls. Living sperm quantity was 2.75 ± 2.47–2.98 and 3.83 ± 3.14–4.62 million in the neonicotinoids and controls respectively (median ± 95% CI).

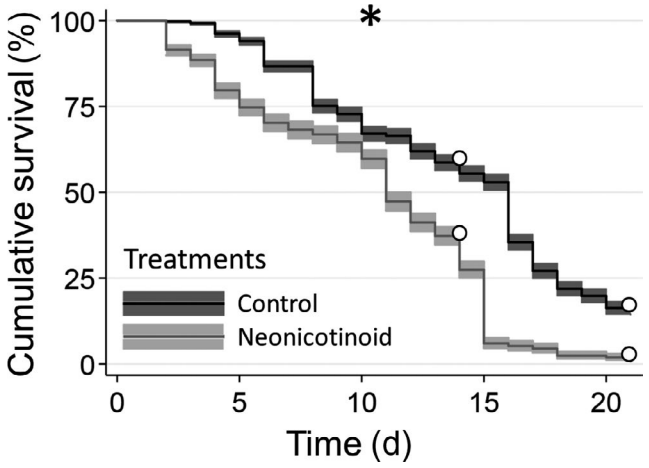

FIGURE 1 Honeybee *Apis mellifera* drone survival curves (Kaplan–Meier). A comparison of cumulative survival between drones under neonicotinoid insecticide exposure ($N = 1,378$) and controls ($N = 1894$) showed a significant difference between treatment groups (multiple effects survival time regression; $p = 0.046$), which is indicated by * ($p < 0.05$). The open white dots indicate points of censorship (e.g. day 14 to assess sperm capacities and day 21 where the experiment was terminated). Shaded areas surrounding the survival curves represent the 95% confidence intervals

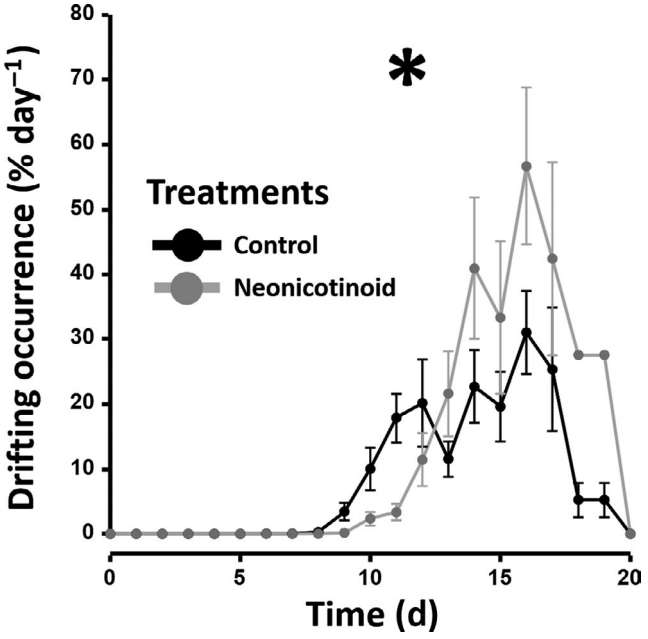

FIGURE 2 Honeybee *Apis mellifera* drone drifting occurrences. Within the first 5 days post-emergence, no drifting was observed for either treatment group; however, drifting was first noted at day 6 by control drones and day 9 by neonicotinoid drones post-emergence. A significant difference was observed in the occurrence of drifting behaviour between neonicotinoid exposed ($N = 1,378$) and control ($N = 1,894$) drones during the evaluated time period (zero-inflated Poisson model, $p = 0.001$) and is indicated by * ($p < 0.001$). Black and grey lines and circles denote observed responses (mean and standard error)

3.4 | Residue analyses

Multiresidue analysis revealed that our test neonicotinoids—thiamethoxam and clothianidin—were not detected in the fresh corbicular pollen used to create our experimental treatment patties (limit of detection - 0.08 ppb and 0.02 ppb, respectively; see Document S1). It also revealed that two other chemicals of the 42 tested were observed above the limit of quantification—acetamiprid at 0.6 ppb and thiacloprid at 0.13 ppb. Three additional substances—methiocarb, fenoxycarb and carbenadazole—were present, but below their limits of quantification (0.5, 0.1, 1.0 ppb respectively). Analysis of our assembled experimental treatment pollen patties revealed 4.3 and 1.1 ppb of thiamethoxam and clothianidin, respectively, in patties fed to colonies belonging to the neonicotinoid treatment group; thiamethoxam and clothianidin were not detected in our control experimental treatment patties.

4 | DISCUSSION

The data demonstrate that exposure to neonicotinoid insecticides can negatively affect reproductive traits of honeybee drones in the field. Employing this approach allowed for novel insight into lethal and sublethal responses under typical field conditions, including effects on behaviour that have not been previously studied in honeybee drones under this context. These efforts revealed that drone survival, physiology and behaviour were all negatively impacted by neonicotinoid exposure. Given the apparent key role of functional males for sexual reproduction, as well as the ubiquitous prophylactic use of agrochemicals worldwide, our results provide a possible mechanism contributing to increased honeybee colony mortality. Our study underscores the need for policy makers to consider inclusion of males in future pesticide risk assessments on bees to provide a more comprehensive, science-based evaluation of their effects on the environment.

Thorough field studies investigating effects of neonicotinoid insecticides on bee species are limited (Blacquière & van der Steen, 2017; Retschnig et al., 2015), primarily because of difficulties in standardizing experiments, susceptibility to variation of space and time within real-world systems and limited resources (Rortais et al., 2017; Simon-Delso et al., 2017; Woodcock et al., 2016). For example, field studies including our own frequently detect residues of common agro-chemicals in their colonies usually occur as a result of worker foraging (Cutler et al., 2014; Raimets et al., 2020; Zioga et al., 2020). This is not surprising given that the typical foraging area of a honeybee colony is ~6 km^2, but can be as high as 150 km^2 under some circumstance (Couvillon et al., 2015). Residue analysis revealed that our tested experimental chemicals (thiamethoxam and clothianidin) were not detected in the fresh corbicular pollen used to make our experimental pollen patties; however, two of the 42 tested chemicals were observed above the limit of quantification—acetamiprid at 0.6 ppb and thiacloprid at 0.13 ppb. Both acetamiprid

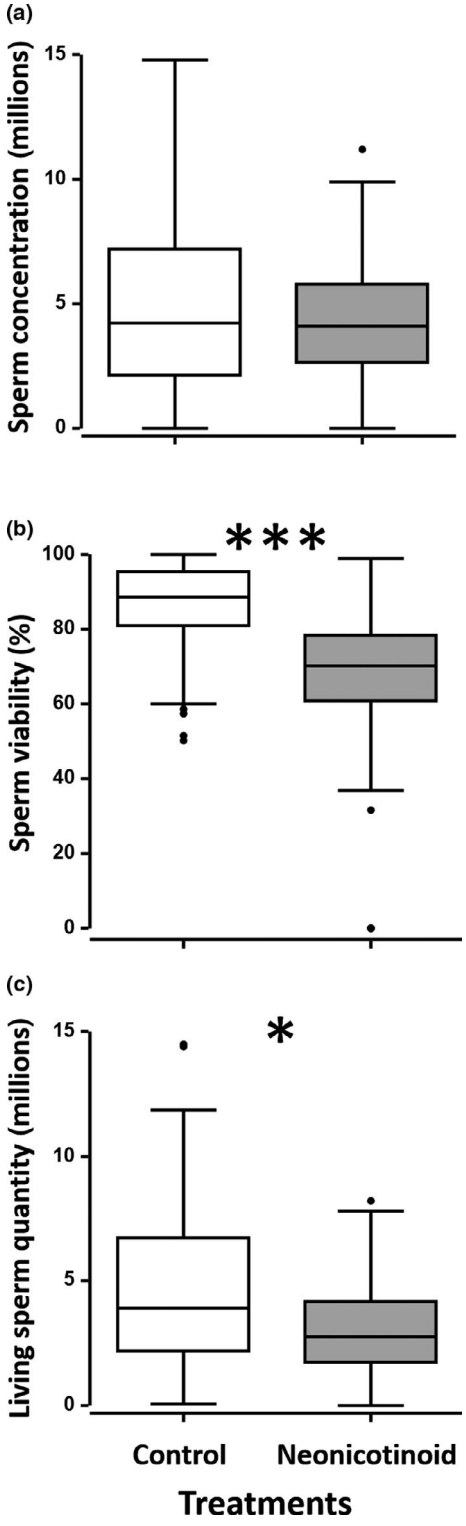

FIGURE 3 Honeybee *Apis mellifera* drone sperm assessment. Assessment of sperm traits in drones under neonicotinoid insecticide exposure ($N = 167$) compared with controls ($N = 169$). (a) Comparison of sperm concentration showed no significant difference (generalized linear mixed models (GLMM); $p = 0.4$). (b) Percentage of viable sperm showed significant differences (GLMM; $p = 0.001$). (c) Living sperm quantity also showed a significant difference between treatment groups (GLMM; $p = 0.031$). All box plots show the interquartile 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 $^{*}p < 0.05$ and $^{***}p < 0.001$

and thiacloprid are relatively less toxic to honeybees (7.14 μg/bee for acetamiprid and 22.59 μg/bee for thiacloprid) than many other common agricultural chemicals, including our tested experimental chemicals—0.0112 μg/bee for thiamethoxam and 0.00739 μg/bee for clothianidin (all oral LD50s; Iwasa et al., 2004; Thompson et al., 2014). We are not aware of any studies that have investigated the influence of acetamiprid and thiacloprid at concentrations observed in our pollen on honeybees. Nevertheless, it is possible that acetamiprid and thiacloprid residues may have interacted with our experimental chemicals, thereby causing the observed effects. It is well known that field studies are more likely to be influenced by external environmental factors that can lead to difficulties in standardizing experiments and interpreting results (Cutler et al., 2014; Rortais et al., 2017; Simon-Delso et al., 2017; Woodcock et al., 2016). More studies are needed to better understand the highly complex web of chemical interactions that honey bee colonies potentially experience in the field (Bird et al., 2021; Gill et al., 2012; Wang et al., 2020). However, we are confident that the experimental design and results are robust, as we used the same corbicular pollen to make both experimental treatment patties and our experimental chemicals (thiamethoxam and clothianidin) were not detected in our controls.

Our study showed that there were no colony-level differences in strength, including the production of drones, between treatment and controls. These results corresponded to some previous field trials (Chauzat et al., 2009; Cutler & Scott-Dupree, 2007; Genersch et al., 2010; Osterman et al., 2019; Rundlöf et al., 2015); however, other studies observed significant colony-level effects as a result of neonicotinoid exposure (Meikle et al., 2016; Sandrock et al., 2014; Stewart et al., 2014; Tsvetkov et al., 2017; Woodcock et al., 2017). Possible differences in observations could be due to the choice of neonicotinoid studied, varying exposure routes, differences in surrounding landscape or underlying honeybee genetics and choice of experimental design features, like number of parameters assessed or duration of assessments. Our work adds to a growing body of literature that employed a particular field-realistic neonicotinoid exposure scenario that so far revealed diverse negative consequences of neonicotinoid exposure to honeybee reproductives (Abdelkader et al., 2019; Ciereszko et al., 2017; Friedli et al., 2020; Grassl et al., 2018; Sandrock et al., 2014; Straub et al., 2016b; Williams et al., 2015). Further work should investigate how local conditions of the experiment, including landscape or weather (Fisher et al., 2018), as well as honeybee genetic diversity (Sandrock et al., 2014), might affect observations and the ability of our results to be generalized to other male bee species.

Drone production can be used as an indicator of overall colony strength (Boes, 2010) and male fitness of colonies (Kraus et al., 2003). We did not observe differences in the number of

immature drones produced, or when they reached adulthood, suggesting that early signs of colony dysfunction as a result of neonicotinoid exposure may be challenging to detect. However, we observed that neonicotinoids significantly influenced the behaviour of adult drones, especially in relation to flight, which could have diverse consequences connected to the movement of individuals among colonies and to mating sites known as drone congregation areas (DCAs; Loper et al., 1992). These observations paralleled other studies that found neonicotinoid exposure influenced memory and behaviour in honeybee workers (Morfin et al., 2019, 2020). Since short-range drifting of honeybees into non-maternal colonies, as observed in our study, is primarily attributed to orientation errors (Currie, 1987; Currie & Jay, 1991; Free, 1956; Moritz & Neumann, 1996; Pfeiffer & Crailsheim, 1998), our findings may correspond to the reduced orientation and flight abilities previously shown in workers exposed to neonicotinoids (Fischer et al., 2014; Tison et al., 2016; Tosi et al., 2017).

Interestingly, we observed a peak period of drifting for both treatment groups around days 14–16. A recent study showed that same period marked when drones started to perform their mating flights (Reyes et al., 2019). It could be that our peak drifting period corresponded to when our experimental drones first performed their long-range orientation or mating flights (Currie & Jay, 1991); perhaps these initial attempts were more prone to orientation errors as the drones learned the spatial relationship of their colony to specific environmental features (Degen et al., 2016; Jay, 1965).

Flight activity of our drones was first recorded in control individuals 6 days post-emergence, whereas neonicotinoid ones showed a 3-day delay. This corresponded to previous studies showing that neonicotinoid insecticides can delay honeybee development and subsequently alter behaviour (Guez et al., 2003; Tavares et al., 2017; Wu et al., 2011). Since short-range orientation flights lasting only a few minutes are crucial to promoting proper sexual development of individuals (Currie, 1982, 1987; Ruttner, 1966), the delay observed in neonicotinoid drone flight highlights how possible developmental effects due to exposure can have important downstream effects that might influence future opportunities of an individual to mate.

At sexual maturity at around 14 days post-emergence, adult drones perform mating flights to specific DCAs (Ruttner, 1956). Our results suggest that neonicotinoid insecticide exposure reduced the longevity of drones by ~50% by this crucial period, in line with previous reports of reduction in drone survival after neonicotinoid exposure (Grassl et al., 2018; Straub et al., 2016a). This severe decrease in longevity could have drastic consequences for both male colony fitness and population-level genetic variation (Neumann & Moritz, 2000; Panhuis et al., 2001). Like previous studies (Reyes et al., 2019; Rueppell et al., 2005; Williams et al., 2015), our method for determining drone longevity employed the assumption that a drone missing from one of our experimental colonies was dead. Indeed, other explanations for drone absence exist, such as drifting to other apiaries. Furthermore, absence may not necessarily imply a negative consequence, since a drone dies after it successfully mates (Gary, 1963). For our experiment, this appears unlikely considering that odds of honeybee drone mating are ~20 000:1 (Page & Metcalf, 1984). While we cannot exclude the possibility that a drone moved to a non-maternal colony in a different location, it is unlikely given the low density of beekeeping in close proximity of our experimental site. In addition, an increased absence of neonicotinoid drones was observed before day 7, when drones typically begin leaving their maternal hive for the first time to perform orientation flights (Currie, 1987). Therefore, despite all possible reasons as to why a drone may be missing, we are confident that the increased absence of drones at the point of sexual maturity is most likely due to mortality.

For those males that did survive to sexual maturity, it is crucial that they transfer high-quality semen (consisting of sperm and mucus) to the oviducts of a queen (Arnqvist & Nilsson, 2000). Insemination quality, therefore, plays an important role not only for honeybee queens but also for the survival of the entire colony as poorly inseminated queens are less attractive to workers and are typically replaced (Abdelkader et al., 2014; Page, 1980). Similar to previous laboratory findings, we confirmed that neonicotinoids can have a significant negative effect on sperm quality (Straub et al., 2016b). This suggests that properly administered laboratory studies can be used as a proxy for understanding some real-world scenarios (Straub et al., 2020). The observation of a reduced number of living sperm in drones 14 days of age could have negative consequences because poorly mated queens are more likely to be replaced (Keller & Nonacs, 1993; Richard et al., 2007; Woyciechowski & Łomnicki, 1987). Further work should investigate how exposure may influence sperm quantity in older drones still capable of mating (Rousseau et al., 2015).

Despite similarities in observations between this study and our previous laboratory effort (Straub et al., 2016b), we intriguingly observed some differences that may shed further light on the influence of experimental test arena. Compared to the laboratory study that saw immatures exposed to experimental treatments and maintained in new laboratory cages (Williams et al., 2013), maintenance of experimental drones exclusively within a colony environment leads to stronger negative effects on mortality and sperm viability by reducing both parameters by roughly 10%. This increase in negative effects observed for drones maintained in colonies may be explained by differences such as increased direct exposure to contaminated pollen as adults in colonies (Mitchell et al., 2017) or increased susceptibility to other stressors within the hive environment, such as microsporidians or viruses (Fries, 1988), that are likely not sources of infection in laboratory cages (Barron, 2015). In contrast, sperm concentration was higher under colony conditions. This may be explained by the ability of drones to fly during our experiment, as flight muscle activation during the first orientation flights can enhance production and maturation of sperm cells, seminal vesicles and mucus glands (Kurennoj, 1954; Ruttner, 1966).

The honeybee queen plays a crucial role in colony cohesion and performance (Moritz & Southwick, 1992; Pettis et al., 2016). Therefore, increased reports of queen failure in Europe and North America have concerned many (Brodschneider et al., 2018b; Kulhanek et al., 2017; Liu et al., 2016). A possible explanation for these recent observations may be the negative effects of neonicotinoid insecticides not on the queens themselves, but possibly on

honeybee drones, as poorly mated queens are more quickly replaced by the workers (Richard et al., 2007). Besides the genetic variance hypotheses, which predicts fitness gains through decreased intracolonial relatedness (Neumann et al., 2000; Palmer & Oldroyd, 2000; Tarpy & Page, 2002), and the sperm limitation hypothesis (Schlüns et al., 2005), which predicts that high levels of polyandry are required to ensure a lifetime supply of sperm for large and long-lived colonies, this provides another adaptive value for extreme polyandry by *Apis* honeybee queens. The number of matings of *Apis* queens can range from a rare single mating under extremely unfavourable environmental conditions (Neumann et al., 1999), to up to 53 (Moritz et al., 1995). Typically, queens mate with 10–20 drones depending on species and local conditions (Haberl & Tautz, 1998; Laidlaw & Page, 1984; Neumann et al., 1999; Palmer & Oldroyd, 2000). This type of mating system may possibly buffer the effects of mating with drones possessing poor quality sperm. However, the consequences for monandrous insect species, ranging from solitary bees to highly eusocial ants (Schläppi et al., 2020), are likely to be much more severe. Obviously, eggs must be fertilized in almost all species; hence, females mate to obtain sperm. Impaired male mating abilities and sperm traits may therefore result in no eggs hatching, or in a male-biased sex ratio depending on life-history and sex-determining mechanism of the species in question.

In conclusion, this study demonstrates that neonicotinoids can adversely affect male honeybee survival, reproductive physiology and behaviour, and therefore provides one plausible mechanistic contributor to increased honeybee colony mortality and decline in other hymenopteran species that exhibit haplodiploidy. This is because haploid males might be more susceptible to environmental stressors because of hemizygosity at key immune and detoxification loci (Friedli et al., 2020; Retschnig et al., 2014). Future studies should more directly investigate how our observed negative effects of neonicotinoids on drone longevity and reproductive physiology impact drone fitness, as well as possible repercussions on queen and colony health. We suggest that policy makers should include males, and not just females, in order to adopt and implement more science-based, holistic risk assessments to comprehensively, yet practically, assess effects of chemicals on important ecosystem service providing insects like the honeybee. Furthermore, we suggest that queen-rearing beekeepers place particular emphasis on queen and drone production management systems that reduce exposure to neonicotinoids, especially during the crucial development stage of their drones, to promote reproductive health of their drones which are so crucial to the overall health of colonies headed by newly mated queens (Moritz & Southwick, 1992; Pettis et al., 2016).

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest.

AUTHORS' CONTRIBUTIONS

L.S., P.N. and G.R.W. designed the experiment and wrote the manuscript; L.S., E.K., L.V.-B., S.B. and J.M. collected field and laboratory data; P.C. and P.N. provided materials and reagents; L.S. and B.V. designed the statistical analysis and wrote the statistical methods and results; L.S., P.C., P.N. and G.R.W. analysed and interpreted the data. All authors contributed to critically revising and editing all versions of the manuscript; they also approved the final version.

DATA AVAILABILITY STATEMENT

Data available via the Dryad Digital Repository https://doi.org/10.5061/dryad.r7sqv9s92 (Straub et al., 2021).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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