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Synergistic and Antagonistic Interactions Between *Varroa destructor* Mites and Neonicotinoid Insecticides in Male *Apis mellifera* Honey Bees

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Pressures from multiple, sometimes interacting, stressors can have negative consequences to important ecosystem-service providing species like the western honey bee (*Apis mellifera*). The introduced parasite *Varroa destructor* and the neonicotinoid class of insecticides each represent important, nearly ubiquitous biotic and abiotic stressors to honey bees, respectively. Previous research demonstrated that they can synergistically interact to negatively affect non-reproductive honey bee female workers, but no data exist on how concurrent exposure may affect reproductive honey bee males (drones). This is important, given that the health of reproductive females (queens), possibly because of poor mating, is frequently cited as a major driver of honey bee colony loss. To address this, known age cohorts of drones were obtained from 12 honey bee colonies—seven were exposed to field-relevant concentrations of two neonicotinoids (4.5 ppb thiamethoxam and 1.5 ppb clothianidin) during development via supplementary pollen patties; five colonies received patties not spiked with neonicotinoids. Artificially emerged drones were assessed for natural *V. destructor* infestation, weighed, and then allocated to the following treatment groups: 1. Control, 2. *V. destructor* only, 3. Neonicotinoid only, and 4. Combined (both mites and neonicotinoid). Adult drones were maintained in laboratory cages alongside attendant workers (1 drone: 2 worker ratio) until they have reached sexual maturity after 14 days so sperm concentration and viability could be assessed. The data suggest that *V. destructor* and neonicotinoids interacted synergistically to negatively affect adult drone survival, but that they interacted antagonistically on emergence mass. Although sample sizes were too low to assess the effects of *V. destructor* and combined exposure on sperm quality, we observed no influence of neonicotinoids on sperm concentration or viability. Our findings highlight the diverse effects of concurrent exposure to stressors on honey bees, and suggest that *V. destructor* and neonicotinoids can severely affect the number of sexually mature adult drones available for mating.

Keywords: honey bee, drone, neonicotinoid, thiamethoxam, *Varroa destructor*, parasite, interaction

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INTRODUCTION

Stressors like habitat loss, climate change, pollution, and invasive species have resulted in widespread anthropogenic effects on ecosystems (Butchart et al., 2010; Geldmann et al., 2014). Research on how multiple stressors interact to pressure important ecosystem-service providing species have revealed complex effects, ranging from additive, whereby the effects of two stressors equal their combined individual effects, to synergism for which the combined effect of two stressors is greater than the predicted additive effects, to antagonism, when the combined effect of two stressors is less than the predicted additive effects (Côté et al., 2016). Mechanisms responsible for each type of interaction vary, but they may be influenced by exploitative competition for limited resources (Poulin, 2007) or host stress as a result of tissue pathology or immune suppression (Alaux et al., 2010; Pettis et al., 2013). Understanding and predicting the circumstances surrounding each type of interaction is currently difficult, but is expected to significantly improve as laboratory, field, and meta-analysis investigations on model or important ecosystem-service providing species continue (Boyd and Brown, 2015; Piggott et al., 2015; Kaunisto et al., 2016).

In recent years, it is believed that pressures caused by multiple, possibly interacting, stressors are responsible for consistently high losses of managed western honey bee (*Apis mellifera*) colonies across the northern hemisphere (Kulhanek et al., 2017; Bruckner et al., 2019; Gray et al., 2019, 2020). Both biotic and abiotic stressors, such as poor nutrition, introduced parasites, heavy metals, and pesticides are blamed (Steinhauer et al., 2018). Individually, they can elicit a range of negative consequences on honey bees, ranging from sub-lethal physiological and behavioral effects to lethal ones that result in reduced survival (Havard et al., 2020). When acting in concert, diverse effects are observed, ranging from antagonistic parasitic-pesticide interactions to synergistic pesticide-pesticide ones (Straub et al., 2020; Bird et al., 2021; Siviter et al., 2021).

Arguably one of the most important biotic stressors for honey bees is the ectoparasitic mite *Varroa destructor* (Rosenkranz et al., 2010; Traynor et al., 2020). Its life cycle is tightly linked to its honey bee host, and consists of two distinct stages—dispersal and reproduction (Traynor et al., 2020). Mature *V. destructor* foundress mites produce several offspring that subsequently feed on tissues of developing honey bees (hereafter called brood) until they emerge from brood cells alongside their honey bee hosts (Rosenkranz et al., 2010; Ramsey et al., 2019). Mature female *V. destructor* mites prefer brood of honey bee males, also called drones, because of specific characteristics such as an extended reproduction period as well as the chemical signals elicited by developing drones (Conte et al., 1989; Fuchs, 1992; Rosenkranz et al., 2010). Mite infestation can negatively affect drone body mass and mating efficiency through increased mortality, reduced flight activity, and low sperm quality (Collins and Pettis, 2001; Duay et al., 2002; Bubalo et al., 2005).

Insecticides like systemic neonicotinoids are important abiotic risk factors (Simon-Delso et al., 2015; Wood and Goulson, 2017). Neonicotinoids are among the most widely applied insecticides globally (Simon-Delso et al., 2015), and are predominantly employed as seed coating treatments translocating throughout the entire plant, including pollen and nectar (Bonmatin et al., 2007; Botías et al., 2015; Pang et al., 2020). Neonicotinoids have been detected in honey bee collected pollen from multiple sources, such as maize (Pilling et al., 2013) and squash (Stoner and Eitzer, 2012), herbaceous plants (Wood et al., 2019), and wild flowers (Botías et al., 2015), as well as in beebread, which is processed pollen stored within the colony (Bonmatin et al., 2015; Mogren and Lundgren, 2016; Tong et al., 2018). Furthermore, these compounds are readily released into the soil and water where they can persist and translocate to adjacent vegetation, posing a risk to non-target organisms (Sur et al., 2003; Bonmatin et al., 2015). The risk of exposure extends from individual honey bee foragers to the entire colony when contaminated resources are shared with other adults and developing individuals (Sanchez-Bayo and Goka, 2014). There is clear evidence that field-relevant concentrations of neonicotinoids have sub-lethal effects on honey bees, eliciting behavioral, physiological, and anatomical changes (Singla et al., 2021). For example, drones experienced reduced development stability and produced fewer living sperm under neonicotinoid exposure, which can ultimately affect colony reproductive potential and overall performance (Straub et al., 2016; Friedli et al., 2020).

Despite *V. destructor* and neonicotinoids considered to ubiquitous stressors to honey bees (Little et al., 2015; Wilfert et al., 2016; Colwell et al., 2017; Mitchell et al., 2017), little is known about their potential interactive effects in honey bees. A handful of studies on non-reproductive honey bee females, the workers, have yielded conflicting results, ranging from no interaction to synergism (Straub et al., 2016; Siede et al., 2018; Morfin et al., 2020; Bird et al., 2021); however, no such work has investigated possible effects on honey bee drones, despite their predicted greater susceptibility to environmental stressors as proposed by the haploid susceptibility hypothesis (O'Donnell and Beshers, 2004), which suggests that drones are likely less resilient to environmental stressors than their worker counterparts due to a lack in allelic variation at important immune related genes (Hamilton, 1964; O'Donnell and Beshers, 2004; Retschnig et al., 2014; Friedli et al., 2020). The availability of high quality drones to mate with reproductive honey bee females, the queens, is crucial for the fitness of those queens and their colonies (Koeniger and Koeniger, 2007), since genetic variation confers benefits such as increased resilience to biotic risk factors (Tarpy, 2003; Tarpy and Seeley, 2006; Delaplane et al., 2015; Simone-Finstrom et al., 2016).

Therefore, for the first time we assessed the effects of simultaneous exposure to neonicotinoid insecticides and the *V. destructor* mite on honey bee drone emergence mass, adult survival, and sperm quality. Based on previous studies that employed worker honey bees, as well as the haploid susceptibility hypothesis, we expected that both stressors individually would have strong negative effects on drones, and that simultaneous exposure would result in a synergistic negative effect (O'Donnell and Beshers, 2004; Blackmon et al., 2015; Straub et al., 2016, 2019; Maher et al., 2019; Morfin et al., 2020).

MATERIALS AND METHODS

Twelve western honey bee (*Apis mellifera*) packages, each headed by a laying sister queen and 1.5 kg workers, were installed in ten-frame Langstroth hives in Auburn AL, United States on 18 March 2020. To promote growth, colonies were provided *ad libitum* with sucrose solution (50% weight/volume with water) for 2 weeks before being randomly assigned to either a control or neonicotinoid treatment.

Neonicotinoid Exposure

Following an established method (Straub et al., 2019; Friedli et al., 2020), neonicotinoid treatments were administered *ad libitum* via pollen patties (60% corbicular pollen, 30% powdered sugar, 10% organic honey), as pollen is a common route of neonicotinoid exposure for honey bees (Wood et al., 2019). The pollen was sourced from corbicular pollen removed from honey bee foragers returning to colonies that were located in a low intensity agricultural region of Colorado; subsequent analysis using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) by the United States Department of Agriculture (USDA) National Science Laboratory (Gastonia, North Carolina, United States) detected no traceable levels of agricultural chemicals (**Supplementary Figure 1**; AOAC International, 2007). As in previous experiments (Straub et al., 2019; Friedli et al., 2020), colonies allocated to the neonicotinoid treatment (n = 7) received pollen patties spiked with field-relevant concentrations of two neonicotinoids—thiamethoxam and clothianidin (4.5 and 1.5 ppb, both Sigma-Aldrich) (Stoner and Eitzer, 2012; Pilling et al., 2013; Botías et al., 2015; Wood et al., 2019). To create these spiked pollen patties, pure analytical standards of both neonicotinoids (purities of > 99%; Sigma-Aldrich®, Burlington, Massachusetts, United States) were dissolved in distilled water (1 mg/L). Aliquots of a single stock solution for each compound were then added to the organic honey, which was then thoroughly mixed by kneading the components of the patties in a large plastic container until a homogenous paste was made (Sandrock et al., 2014). Concentrations were confirmed by the USDA National Science Laboratory (Gastonia, North Carolina, United States) using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) (AOAC International, 2007) 4 ppb for thiamethoxam and < 6 ppb for clothianidin; levels of detection were 1 and 6 ppb, respectively) (**Supplementary Figure 2**). The control treatment (n = 5) was fed non-neonicotinoid spiked pollen patties. Prior to feeding, each colony was equipped with a Sundance pollen trap (Rossman Apiaries, LLC., Moultrie, GA, United States) to promote in-hive patty consumption and prevent the influx of natural pollen (Sandrock et al., 2014; Williams et al., 2015). Following a previously employed feeding regime (Forfert et al., 2015; Williams et al., 2015; Straub et al., 2019), pollen patties were provided for 49 days to cover two entire brood cycles (Winston, 1991), and to mimic a realistic exposure period encountered by foraging honey bees. Earlier studies have demonstrated that foraging honey bees can be exposed to neonicotinoid residues for a similar period because of overlapping bloom periods of treated crops (Tsvetkov et al., 2017), contaminated planter dust that is exhausted to the environment (Krupke et al., 2012), and crops and neighboring non-agricultural foraging areas being contaminated as a result of leaching (Botías et al., 2015; Schaafsma et al., 2015; Long and Krupke, 2016; Mogren and Lundgren, 2016).

Experimental Drones

To obtain a known age cohort of drones for the experiment, at 42 days post initial neonicotinoid exposure the queen of each colony was caged for 2 days onto a drone brood frame previously drawn-out by her own colony during neonicotinoid exposure (Williams et al., 2013). These drone frames remained in their respective colonies for 20 days more. Subsequently, the frames were moved on day 22 to an incubator (34°C and 60% RH, DR-41NL, Percival Scientific, Inc., Perry, IW) (Williams et al., 2013). The next day, which was 1 day prior to expected natural emergence (Winston, 1991), drones were artificially emerged from the capped brood cells using forceps. Each brood cell and drone were visually inspected for *V. destructor* parasitism, defined as the observation of one or more adult female *V. destructor*. Each drone was also inspected for wing deformities, which are typically clinical symptoms of deformed wing virus (Dainat et al., 2012), weighed to the nearest 0.1 mg using an analytical scale (VWR B2-Series, VWR, Radnor, PA, United States), and then assigned to one of the four treatment groups based on their previous colony-level exposure to neonicotinoids and individual *V. destructor* parasitism status (Straub et al., 2019): (1) No neonicotinoids and No *V. destructor* (Control), (2) No neonicotinoids and Yes *V. destructor* parasitism (*V. destructor* only), (3) Yes neonicotinoids and No *V. destructor* parasitism (Neonicotinoid only), and (4) Yes neonicotinoids and Yes *V. destructor* parasitism (Combined).

Drone Survival and Sperm Quality

For each colony, we established up to five hoarding cages per treatment group from each available colony which resulted in a total of 60 experimental cages (Williams et al., 2013). Cages were made of 250 cm^3 plastic cups (Plastikbecher.de GmbH, Giengen, Germany) equipped with a round lid. A ventilation hole (6 cm in diameter) was cut out from the lid and covered with a felt cloth (Maier Haushaltspflege GmbH, Murg, Germany). Generally, each cage contained 10–12 adult drones and 20–24 adult workers from the same colony. Adult workers were collected from a brood frame and added to the cage to provide caretaking duties for the drones (Ruttner, 1966; Straub et al., 2016). If a colony did not yield 10 drones for each cage we nonetheless collected all available drones. Therefore, we did not obtain 10 drones for nine of the 60 cages. Regardless, the adult drone to worker ratio was maintained at 1:2 for all cages for the duration of the experiment. Hoarding cages were kept in the incubator (30°C and 60% RH, DR-41NL, Percival Scientific, Inc., Perry, IW) and equipped with a 5 ml syringe containing sucrose solution (50% w/v) to feed the honey bees (Williams et al., 2013). Additionally, a 1.5 ml polypropylene tube (Eppendorf, Enfield, CT, United States), modified to act as an in-cage feeder containing sucrose solution (50% w/v), and a 1.5 ml polypropylene tube, modified to act

as an in-cage feeder containing pollen (60% corbicular pollen, 40% powdered sugar), were added to facilitate autonomous drone feeding and to promote development and maturation of male reproductive organs, respectively (Brodschneider and Crailsheim, 2010; Williams et al., 2013). All food resources were provided *ad libitum*; feeders were replaced every 4 days (Fryday et al., 2015; Minnameyer et al., 2021). An orientation flight was simulated 8 days post cage initiation by exposing cages to indirect sunlight for 24 h; this has been suggested to mark the completion of sperm transition from testis to the seminal vesicles and initiate full maturation of the ejaculate (Schlüns et al., 2005; Hayashi and Satoh, 2019). Adult drones were maintained in hoarding cages for 14 days post emergence, when all surviving individuals were expected to be sexually mature (Rhodes et al., 2011); dead drones and workers were removed daily from each cage.

All individuals surviving to day 14 post emergence were then sacrificed for subsequent *in vivo* sperm quality assessments to prevent sperm migration from seminal vesicles to the bulb (Straub et al., 2016). In brief, the abdomen of each drone was detached from its thorax using dissection scissors, then pinned onto a wax plate before removing ventral sternites so that the testes, mucus glands, and seminal vesicles could be removed using a forceps. For each individual, all structures were placed in a 1.5 ml polypropylene tube (Eppendorf, Enfield, CT, United States) containing 500 μl $Kiev^+$ buffer and crushed to make a diluted sperm stock solution (SSS) (Carreck et al., 2013). Subsequently, sperm viability and concentration were assessed following Straub et al. (2016, 2021). For sperm viability, a 50 μl aliquot of the SSS was added to a 1.5 ml polypropylene tube containing 50 μl $Kiev^+$ buffer (Company, City, State, United States). Then, 2 μl of Hoechst 33342 (0.5 mg ml^{-1}) and 1 μl of propidium iodide (1 mg ml^{-1}) (both Sigma-Aldrich®, Burlington, Massachusetts, United States) were added to label living (viable; green) and dead (non-viable; red) sperm. The suspension was then incubated for 20 min in complete darkness and then gently vortexed. Next, 10 μl of the solution were examined on a microscope slide at 400x magnification using a fluorescent light microscope (Leica, DM2500 LED, Morrisville, NC, United States). Ten arbitrary visual fields were selected to count the quantity of viable and non-viable sperm; an average value was then calculated from these fields. For sperm concentration, 20 μl SSS were diluted with 80 μl $Kiev^+$ buffer (1:5 dilution) in a 1.5 ml polypropylene tube, then an aliquot was transferred to a cell counting chamber (Thermo Fisher Scientific, Waltham, MA, United States) to count sperm using the fluorescent light microscope. Total sperm concentration (in 500 μl SSS) was calculated by multiplying the average number of sperm counted in two chambers by the dilution factor (1:5) by the volume used for the counting chamber (10 μl) by the SSS volume (500 μl). Lastly, living sperm concentration was determined by multiplying average sperm viability by total sperm concentration.

Colony Parameters

Just prior to queen caging at 39 days post initial neonicotinoid exposure, adult bees and capped brood (bees developing under a wax capping) were visually assessed in each colony using the Liebefeld estimation method (Delaplane et al., 2013). For this, both colony strength parameters were first estimated for each frame in each colony as a percentage of frame coverage from 0 to 100. Then, percent coverage was converted to an absolute value of number of bees and area (cm^2) for adults and capped brood, respectively. Colony-level *V. destructor* infestation was assessed at the same time using the alcohol wash method to determine the number of mites per 100 adult bees by sampling ∼300 adult bees from the brood nest (Dietemann et al., 2013).

Statistics

All statistical analyses were performed in R (version 4.0.2., 11/2/20) using a significance level of $\alpha = 0.05$. Colony-level neonicotinoid exposure and individual-level *V. destructor* infestation were always contained as fixed factors in the model, like (Straub et al., 2019). Employing a backward selection approach, we built linear Mixed Effect Models (lmm) for normally distributed data and Generalized Linear Mixed Effect Models (glmm) for data that was not normally distributed; data were tested for normality using the *ggdensity* and *ggqqplot* function from the R package *ggpubr*. All models started as a full model including all explanatory variables that could potentially affect observed variation in response variables (e.g., sperm viability). When a significant effect was detected, explanatory variables were included as random factors. The significance of individual explanatory variables was assessed using the Akaike Information Criterion (*AIC* function in R). Based on this approach, a lmm was built using the *lmer* function from R package *lme4* to assess effects of neonicotinoids and *V. destructor* parasitism on emergence body mass of drones, while including colony and cage identification number as random factors.

The R package *survival* was used to fit a Cox proportional hazards regression model using the *coxph* function to assess effects of individual and combined neonicotinoid exposure and *V. destructor* infestation on drone survival. Furthermore, survival curves (Kaplan-Meier plots) were plotted using the *ggsurvplot* function and hazard rates for each treatment group and calculated using the *tbl_regression* function. Subsequently, cumulative survival was compared between treatment groups by using the *pairwise_survdiff* function from the R package *survminer* which allowed for pairwise comparisons with a Bonferroni correction [Survival Bonferroni Multiple Comparison Test (sbmct)].

For sperm concentration and living sperm concentration, a glmm including cage identification number as random factor to account for potential clustering effects was built. Data were not transformed. Therefore, a best fit distribution was incorporated (family = Poisson). For sperm viability data, a l mm was fitted to assess fixed factor effects, with cage and drone identification numbers as a random factors to account for potential clustering effects. For each model, *post hoc* pairwise comparisons of all treatment groups were performed by using the *lsmeans* function from the *emmeans* package in R and using a Bonferroni correction for multiple comparisons [Bonferroni Multiple Comparison Test (bmct)]. To identify potential interactions between neonicotinoid exposure and *V. destructor* infestation, an additive effects framework was employed (Folt et al., 1999). Interactions were considered synergistic or antagonistic if the

effect of the combined stressor treatment group was greater or smaller than the sum resulting from individual stressors (*V. destructor* and neonicotinoid) (Hay, 1996). To assess this, the percent difference in treatment groups compared to the controls were calculated using mean survival [d], total sperm quantity [#], living sperm quantity [#], and sperm viability [%].

To assess the effects of the colony-level neonicotinoid treatment on number of adult bees, a glmm was built with a best fit distribution (family = negative binomial) to account for overdispersed count data. Similarly, a glmm with a best fit distribution (family = poisson) was fitted to assess effects on colony-level *V. destructor* infestation. Both glmm's were followed by a Wilcoxon Rank Sum Test (wrt) using the *wilcox.test* function. For capped brood area, a lmm was fitted, followed by a Two-Samples *t*-test (*t*-test) using the *t.test* function.

RESULTS

Colony Parameters

No significant differences were observed between neonicotinoid treated colonies and control colonies for number of adult bees (wrt, $W = 90$, $p = 0.42$), capped brood area (*t*-test, $t = 0.54$, $p = 0.59$), or *V. destructor* mite infestation (wrt, $W = 11$, $p = 0.19$) (**Supplementary Table 1**). The *V. destructor* count per 100 bees for neonicotinoid treated colonies was 1.5 ± 1.6 (median $\pm$ SD) compared to 1 ± 0.5 (median $\pm$ SD) for control colonies. Wing deformities were not observed for any of the newly emerged drones.

Drone Emergence Body Mass

Body mass of newly emerged drones ($n = 792$) was significantly affected by both neonicotinoid exposure (Linear Mixed Effect Model (l mm), $t = 2.99$, $p = 0.003$) and *V. destructor* infestation (lmm, $t = -7.20$, $p < 0.001$) (**Figure 1** and **Supplementary Table 1**). Compared to drones from the Control ($n = 247$), *V. destructor* only ($n = 91$) and Combined stressor ($n = 86$) treatment groups, emergence body mass was significantly higher in Neonicotinoid only drones ($n = 368$, mean $\pm$ SE, 274.2 ± 3.9 mg; Bonferroni Multiple Comparison Test (bmct), *all* $p < 0.05$). The lowest body mass was recorded in *V. destructor* only drones, which was significantly different from the Control (mean $\pm$ SE, 245.9 ± 4.4 mg; bmct, $p < 0.001$) and Combined treatment groups (bmct, $p = 0.02$). Body mass of Control (mean $\pm$ SE, 261.8 ± 4.3 mg) and Combined drones (mean $\pm$ SE, 258.3 ± 4.3 mg) did not differ from each other (bmct, $p > 0.05$) (**Supplementary Tables 2, 3**). Exposure to the Combined stressors reduced worker body mass by 0% compared to the Controls. This was higher than the sum of the individual stressors, which was −2.1% because of a 4.0% increase for Neonicotinoid only and 6.1% reduction for *V. destructor* only treatment workers. This suggests an antagonistic interaction.

Drone Survival

Significant differences in adult drone survival were observed among treatment groups (**Figure 2**). Although no difference in survival was observed between *V. destructor* only and Control drones (sbmct, $z = 1.96$, $p = 0.16$), Neonicotinoids only (sbmct, $z = 2.84$, $p = 0.03$) and Combined drones (sbmct, $z = 6.61$, $p < 0.001$) experienced significantly reduced survival compared to Controls (**Supplementary Tables 2, 3**). Despite the lack of a statistical difference, hazard rate (HR) for *V. destructor* only drones was 135% (95% CI [100, 182%]) compared to Controls, whereas it was 136% (95% CI [110, 168%]) and 267% (95% CI [200, 358%]) for drones belonging to Neonicotinoid only and Combined treatment groups, respectively (**Supplementary Table 4**). Compared to Controls, the reduction in survival of adult drones that were exposed to Combined stressors (55%) was greater than the sum of individual stressor effects, which were 17 and 28% reductions for Neonicotinoid only and *V. destructor* only drones, respectively. This suggests a synergistic interaction between the two stressors. This is further supported by the increase in HR for the Combined stressors (167%) compared to Controls, which was also greater than the sum of increased HRs for individual stressors, which was 35% for *V. destructor* only and 36% for neonicotinoid only.

Sperm Quality

Compared to Controls, Neonicotinoid only drones did not experience a significant reduction in sperm concentration [Kruskal-Wallis rank sum test (KW), $\chi^2 = 2.11$, $p = 0.15$], sperm viability (*t*-test, $t = 1.72$, $p = 0.09$), or living sperm concentration (KW, $\chi^2 = 0.69$, $p = 0.41$) (**Supplementary Tables 1, 3**). Sample sizes of drones belonging to the *V. destructor* only and the Combined treatment group were lower than 15 for each sperm quality trait. Therefore, these two treatment groups were omitted from statistical analyses regarding sperm quality traits.

DISCUSSION

Pressure caused by stressor interactions is believed to be responsible for widespread negative effects on biodiversity (Butchart et al., 2010; Barnosky et al., 2011), including on the economically and ecologically important honey bee (Alaux et al., 2010; Straub et al., 2019). For the first time, we investigated the potential effects of simultaneous pressure from neonicotinoid insecticides and *V. destructor* mites on adult male honey bee (drone) emergence mass and survival. The data revealed that combined exposure to both stressors resulted in a negative synergistic effect on adult drone survival, but an antagonistic effect on emergence mass. Our results suggest that combined exposure to these two ubiquitous stressors can severely affect the availability of drones for mating, and further highlight the complexity of potential interactive effects of simultaneous stressor pressures on honey bees.

Effects of concurrent exposure from multiple stressors on honey bees have garnered considerable attention of late. This is especially the case considering negative synergistic interactions for which the effect of multiple concurrent factors are worse than the sum of individual effects (Maher et al., 2019). As hypothesized, we observed a synergistic effect of neonicotinoids and *V. destructor* on adult drone survival. Interestingly, this

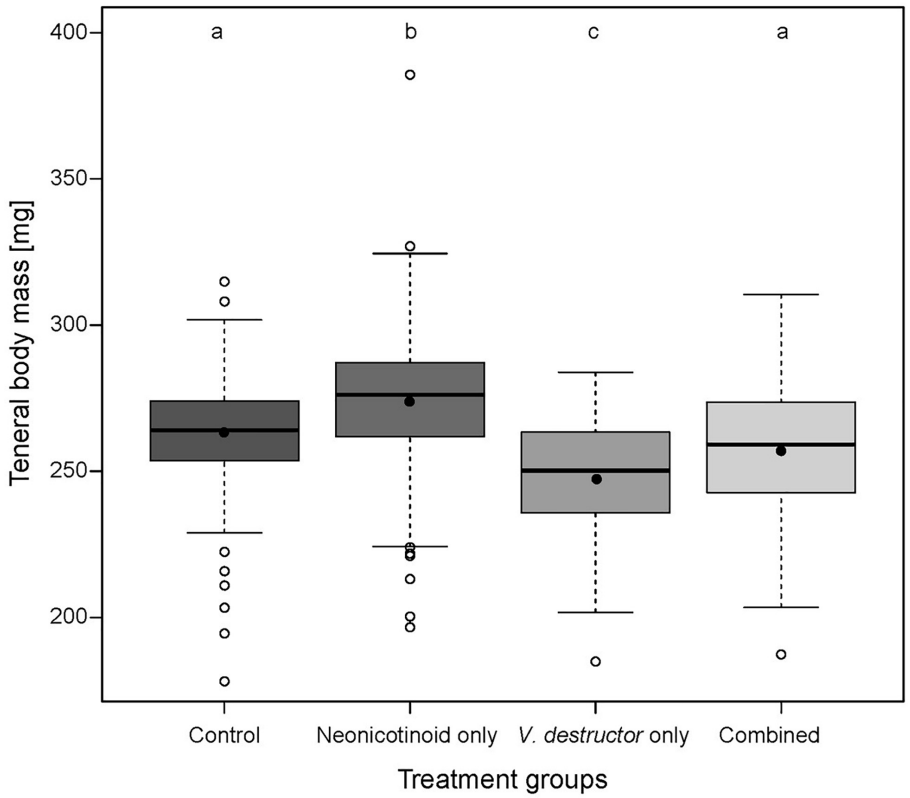

FIGURE 1 | Emergence body mass of experimental western honey bee (*Apis mellifera*) drones across four treatment groups: Control—drones without neonicotinoid exposure and without *Varroa destructor* parasitism (n = 247), Neonicotinoid only—drones exposed to neonicotinoids during development but free from *V. destructor* parasitism (n = 368), *V. destructor* only—drones infested by *V. destructor* during development, but not exposed to neonicotinoids (n = 91), and Combined—drones exposed to both neonicotinoids and *V. destructor* during development (n = 86). Boxplots show the inter-quartile range (box), the mean (black circles), the median (black line within box), data range (vertical black lines from box), and outliers (open circles). Different letters above boxplots indicate statistically significant differences ($p \leq 0.05$).

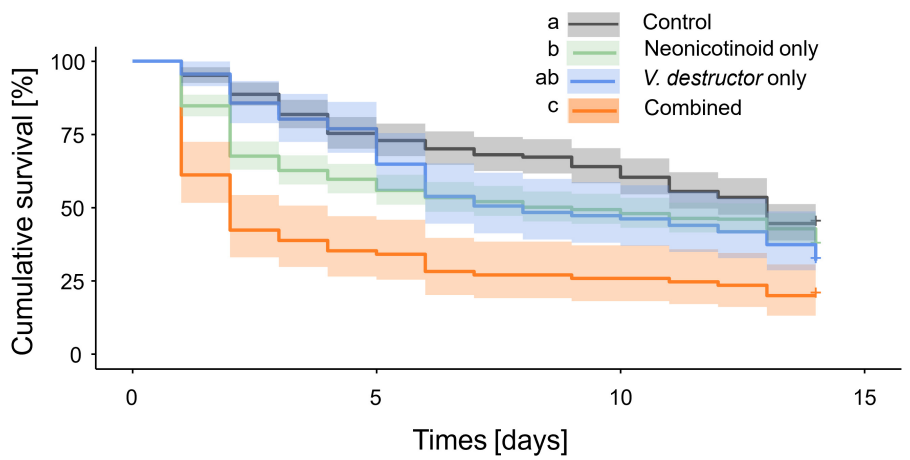

FIGURE 2 | Kaplan-Meier survival curves of experimental western honey bee (*Apis mellifera*) drones during a 14-day laboratory cage assay. Summary of survival curves for all four treatment groups: Control—drones without neonicotinoid exposure and without *Varroa destructor* parasitism, Neonicotinoid only—drones exposed to neonicotinoids during development but free from *V. destructor* parasitism, *V. destructor* only—drones parasitized by *V. destructor* during development but not exposed to neonicotinoids, and Combined—drones exposed to both neonicotinoids and *V. destructor* during development. Different letters placed before treatment groups indicate statistically significant differences ($p \leq 0.05$).

same interaction was not observed for adult worker survival by a similar experiment performed during the same time of year (Straub et al., 2019). Although that study was performed in a different location, comparative results between the two suggest that differences in observed combined stressor effects on adult drone and worker survival could be explained by the haploid susceptibility hypothesis. This hypothesis states that drones experience higher susceptibility to environmental stressors due to their hemizygosity at loci involved in immune response (O'Donnell and Beshers, 2004; Blackmon et al., 2015). Since both neonicotinoids and *V. destructor* are known to impair the immune response of honey bees (Claudianos et al., 2006; Prisco et al., 2013; Brandt et al., 2016), a reduction in allelic diversity due to hemizygosity may result in more severe consequences when exposed, although future studies should be performed under the same conditions to further explore this as a reason for differences observed between these two types of honey bees.

In contrast to the observed synergistic negative effect on adult drone survival, we found that the two stressors had an antagonistic effect on drone emergence mass. Similar, contradictory observations were observed by a recent meta-analysis of agrochemical pesticide interactions, whereby proxies of honey bee fitness like behavior and physiology revealed additive or antagonistic effects, whereas synergistic effects were common on honey bee mortality (Siviter et al., 2021). A possible explanation for our observation may be because neonicotinoids are known to affect honey bee carbohydrate and lipid metabolism (Derecka et al., 2013; Cook, 2019). For example, Cook (2019) found that honey bees exposed to low concentrations of clothianidin had high lipid contents compared to controls. Thus, feeding by *V. destructor* during development may possibly offset the gain in body mass resulting from metabolic dysfunction associated with exposure to neonicotinoids, since *V. destructor* parasitism alone reduced emergence body mass in our experiment. Unfortunately, due to low sample size as a result of synergistic effects on mortality, we could not sufficiently examine possible interactions of neonicotinoids and *V. destructor* on sperm traits. Although examining all stressor combinations on multiple proxies of honey bee fitness are not realistic (Côté et al., 2016), the synergistic interaction that we observed on drone mortality by the time of sexual maturity could justify further attempts to understand how these two ubiquitous stressors affect the reproductive health of drones at this important time period, as a reduction in queen health, possibly due to poor mating, is frequently reported as a primary reason for honey bee colony mortality (Pettis et al., 2016).

Both neonicotinoids and *V. destructor* have each been shown to negatively affect drone health and performance (Rangel and Fisher, 2019; Friedli et al., 2020; Straub et al., 2021). On its own, *V. destructor* parasitism reduced drone emergence body mass by 6% compared to controls during our experiment. This has been shown for both workers and drones (Bowen-Walker and Gunn, 2001; Duay et al., 2003; Blanken et al., 2015; Ramsey et al., 2019; Straub et al., 2019), and is likely the result of mites feeding on the honey bees' fat bodies during development. In contrast, exposure to neonicotinoids alone resulted in increased emergence body mass of drones (+ 4%). Low concentrations of neonicotinoids are known to affect carbohydrate and lipid metabolism, both which could affect body mass (Derecka et al., 2013). Other examples suggest that hormesis, a biphasic dose response in which biological processes can be stimulated by low concentrations of normally harmful chemical (Calabrese, 2005), can result in increased body mass, possibly at the expense of fitness (Cutler, 2012; Cutler and Rix, 2015). Indeed, our results suggest a negative effect of neonicotinoids on drone survival until sexual maturity, 14 days post emergence. A possible explanation for this could be a reduction in detoxification-competence (Claudianos et al., 2006). Additionally, experimental drones might have been nourished by compromised worker nurses during development, as previous investigations demonstrated that neonicotinoid exposure negatively affected nurse food glands. Additionally, experimental drones might have been nourished by compromised worker nurses during development, as previous investigations demonstrated that neonicotinoid exposure negatively affected nurse food glands (Hatjina et al., 2013). In contrast, *V. destructor* parasitism had no effect on adult drone survival. This is unexpected, since mites prefer drone brood cells and have been shown to negatively affect adult worker survival (Straub et al., 2019). Surprisingly few efforts have investigated the effects of *V. destructor* on drone survival (Fuchs, 1992; Rinderer et al., 1999; Collins and Pettis, 2001). For example, Collins and Pettis (2001) found that survival of drones parasitized by more than one reproductive female mite was not impacted when only one female *V. destructor* was present. Thus, low levels of colony-level infestation in our study could explain why we also did not observe a negative effect of *V. destructor* alone on drone survival. Despite a lack of a significant reduction in drone survival, it is noteworthy that *V. destructor* parasitism during development still increased the hazard ratio for drones (35% compared to Controls, **Supplementary Table 4**); thus, parasitized drones still likely experience increased stress. We were not able to assess effects of *V. destructor* only on sperm quality traits due to low sample size at the end of the cage trial. Relative to the Control and Neonicotinoid only treatment groups, fewer drones were assigned to this treatment groups at the start of the trail, potentially because parasitized developing drones were removed from their cells prior to artificial emergence (Harbo and Harris, 2005). Low adult survival of drones exposed to *V. destructor* during development further limited sample size. However, we found that there was no effect of neonicotinoids on sperm quality traits when drones were exposed during development. These results align with Ciereszko et al. (2017), but they contrast Straub et al. (2016) who found negative effects on sperm viability and concentration of living sperm, but not total sperm concentration. Given that spermatogenesis begins during the larval stage and terminates at pupation (Yániz et al., 2020), drones were potentially only exposed to a low dose of insecticides for a portion of the total spermatogenesis process, during the larval stage. This highlights that testing arena and experimental design likely contribute to the complexity of comparing observations among multiple studies, and demonstrates the importance of controlled ring tests performed in multiple laboratories when investigating stressor effects (Medrzycki et al., 2013; van der Sluijs et al., 2015).

Our results highlight that interaction effects between two important stressors, like *V. destructor* mites and neonicotinoid insecticides for honey bees, can range from synergism to antagonism depending on the variable measured. According to our findings, neonicotinoids and *V. destructor* interacted synergistically to induce a severe lethal effect on adult honey bee drones, but interacted antagonistically on drone emergence body mass. The complexity of stressor interactions in the honey bee warrants future work elucidating outcomes of simultaneous pressure from multiple stressors, especially for risk assessment schemes that may otherwise not accurately estimate the interactive effects of stressors. Additionally, extrapolating effects observed under laboratory conditions to the field remain a major challenge and additional field-studies are required to confirm our findings.

DATA AVAILABILITY STATEMENT

Data are available via the Dryad Digital Repository at https://doi.org/10.5061/dryad.gmsbcc2p8.

AUTHOR CONTRIBUTIONS

SB, GW, and PN developed the experimental design. SB performed the experiment and collected the data. SB and LS performed statistical analysis. All authors wrote the manuscript and approved its final submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.756027/full#supplementary-material

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