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ORIGINAL RESEARCH ARTICLE

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The influence of sub-lethal neonicotinoid doses and ambient temperature on individual *Apis mellifera scutellata* flight efficiency

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ABSTRACT

Honey bee (*Apis mellifera*) thermoregulation plays an integral part in their behaviour and physiology and has been shown to be vulnerable to the effects of neonicotinoid insecticides. Flight muscles are a crucial source of physiological heat as well as being vital for behavioural heat regulation, and are negatively affected by neonicotinoid insecticides. In this study, we evaluated the flight efficiency and capacity of *Apis mellifera scutellata* under the influence of both elevated ambient temperatures and sublethal neonicotinoid exposure. The various aspects of flight; success, distance, speed, and duration, were not notably affected by these factors. However, the honey bees' ability to initiate a successful flight was significantly affected by neonicotinoid exposure. Such a reduction in honey bee flight capacity, and flight muscle function in general, especially under the increasing frequency and intensity of hot weather events, is cause for concern when considering legislation and use of these neonicotinoids in the agricultural and suburban setting.

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Introduction

Among the multitude of negative effects that insecticide use has on target pests as well as non-target beneficial arthropods such as honey bees, is an influence on a variety of health and foraging-related factors. Foraging honey bees come into contact with a multitude of pesticides in the environment (Mullin et al., 2010; Prado et al., 2019; Samson-Robert et al., 2014; Woodcock et al., 2017) and the residues of these pesticides have been identified in a wide spectrum of food sources for both honey bees and humans alike (Chen et al., 2014; Lu et al., 2016; Mitchell et al., 2017).

Chronic exposure to insecticides impairs honey bee optomotor behaviour and by extension, foraging behaviour (Parkinson et al., 2022). Of particular concern are the neonicotinoid insecticides and their metabolites, which act as agonists of the nicotinic acetylcholine receptors of insects (Simon-Delso et al., 2015). In contrast to the strongly regulated agricultural landscape of the USA, the United Kingdom and the European Union, the extent of use of these neonicotinoid pesticides on the African continent is not well documented. The effects that neonicotinoids have on pollinators and ecosystems in Africa as a whole cannot be directly inferred from the wide range of research and documented findings from across the EU, UK and North America. Neonicotinoid pesticides constitute around 25% of global insecticide sales (Jeschke et al., 2011; Simon-Delso et al., 2015), with imidacloprid, clothianidin and thiamethoxam specifically accounting for almost 85% of total neonicotinoid sales for use in crop protection in 2012 (Bass et al., 2015). In recent years there has been increased pressure to ban the use of these three neonicotinoids due to mounting global concern about their effects on honey bees. The metabolites of neonicotinoids affect a variety of neurological functions including key learning and foraging behaviours (Frost et al., 2013; Schneider et al., 2012; Teeters et al., 2012; Williamson & Wright, 2013) and lowered immune system resistance by suppressing immunity-related genes and increasing susceptibility to infection by viruses and pathogens (Alaux et al., 2010; Aufauvre et al., 2012; Brandt et al., 2016; Doublet et al., 2015; Sánchez-Bayo et al., 2016; Vidau et al., 2011). Exposure to the neonicotinoid imidacloprid induces rapidly neurotoxic symptoms, including movement coordination difficulty, trembling and tumbling (Suchail et al., 2000). Similarly, sublethal doses of the neonicotinoids

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thiamethoxam, imidacloprid and acetamiprid can impair bee behaviour and motor functions (Aliouane et al., 2009; Charreton et al., 2015; Lambin et al., 2001; Williamson et al., 2014). As a result of these compromised neurological functions, foraging activities vital for colony survival and reproduction are also negatively impacted (Gill et al., 2012; Schneider et al., 2012; Scholer & Krischik, 2014; Yang et al., 2008). For example, exposure to imidacloprid hampers honey bee pollen collection efficiency, which in turn results in more honey bee workers being recruited for foraging at a younger age (Colin et al., 2019) at the expense of sufficient workers available for brood care (Gill et al., 2012). This may not only result in insufficient colony pollen stores but also lowered worker production and a further impaired colony workforce (Blanken et al., 2015; Gill et al., 2012).

Neonicotinoid-induced neurological impairment affects foraging through various aspects of flight capacity including flight distance, duration, velocity, and efficiency (Blanken et al., 2015; Ma et al., 2019; Tosi et al., 2017) and metabolic energy availability for flight muscles (Nicodemo et al., 2014). Imidacloprid exposure reduces flight capacity in honey bees, and even more so when acting together with the parasitic mite *Varroa destructor* (Blanken et al., 2015). Flight performance depends on thoracic muscle activity, and flight muscle temperature is precisely controlled by honey bees during flight (Esch, 1988; Schmaranzer, 2000; Stabentheiner, 2001). Apart from the mechanical action of flight, honey bees use their thoracic muscles to produce heat (Esch, 1976) and impaired individual thermoregulatory capability could be caused by the effect of the pesticide on thoracic muscle activity. *Apis mellifera scutellata* thorax temperature was affected by thiamethoxam exposure for at least 24 h, likely as a result of affected flight muscle function (Tosi et al., 2016). Non-flight thermogenesis, found to be negatively affected by dietary neonicotinoids in bumble bees (*Bombus terrestris*) (Potts et al., 2018), is crucial for pre-flight warm-up (Esch et al., 1991; Heinrich, 1975; Krogh & Zeuthen, 1941).

Environmental temperature is also important for honey bee flight, including the efficiency with which they forage at certain temperatures and the onset and termination of daily foraging activity (Tan et al., 2012). The relatively constant thermal environment within a honey bee hive is advantageous in that it enables workers to commence foraging activities earlier in the day than other stingless or solitary bee species (Heinrich, 1981). Flight muscle shivering is employed to elevate thoracic temperature to suitable levels to facilitate flight, typically between 36 °C and 38 °C in *Apis mellifera* foragers (Heinrich, 1979), and capable of maintaining this elevated T_{th} even at low environmental temperatures (<20 °C) (Dyer & Seeley, 1987; Heinrich, 1979). However, a negative correlation has been found between foraging and flight activity and elevated ambient temperatures in *A. m. carnica* and *A. m. jemenitica* (Abou-Shaara et al., 2013; Blazyte-Cereskiene et al., 2010). Foragers are at particular risk of environmental neonicotinoid exposure and the nature of their foraging tasks means they are also exposed to the widest range of ambient temperatures. Nectar and pollen foragers complete an average of around 10 trips a day (Winston, 1987), although there is a great deal of plasticity surrounding foraging capacity, based on various environmental and societal factors (Tenczar et al., 2014). These foragers are thus at risk of oral exposure to residues in nectar as well as contact exposure with treated plants (Koch & Weißer, 1997) through pollen during pollen and nectar foraging (Louveaux, 1958; Parker, 1981) and adsorption of contaminated dust particles (Prier et al., 2001). They are also at risk of affecting the whole colony with contaminated pollen and nectar (Bos & Masson, 1983; Villa et al., 2000), a risk which is amplified in the case of systemic insecticides, i.e., neonicotinoids (Waller et al., 1984). Water collection is integral to evaporative cooling activities, and the presence of neonicotinoid contaminated surface water (Samson-Robert et al., 2014; Schaafsma et al., 2015; Starner & Goh, 2012) poses an additional risk to water foragers, and by extension the colony as a whole (Samson-Robert et al., 2014; Schaafsma et al., 2015; Simon-Delso et al., 2015). Honey bees are exposed to multiple and overlapping neonicotinoid sources and it is important to establish their effects. Previous studies have demonstrated the negative impact of neonicotinoid exposure on honey bee flight (Blanken et al., 2015; Tosi et al., 2017).

In this study, we aimed to further evaluate the effects of exposure to sublethal doses of three commonly used neonicotinoids, clothianidin, imidacloprid and thiamethoxam, on several aspects of flight ability under varying ambient temperature conditions, for the African honey bee subspecies *A. m. scutellata* (Lepeletier). We did so by recording tethered flights using flight mills to quantify flight success, number of flights, flight distance and flight speed. We predicted that exposure to sublethal doses of neonicotinoids would decrease the tethered flight ability of honey bees and that this effect would be most apparent at lower temperatures due to potentially reduced capacity for flight muscle shivering.

Materials and methods

Study species

The study ran from October 2016 to August 2017, using workers of African honey bees, *A. m. scutellata*, from three healthy experimental colonies housed at the Social Insects Research Group (SIRG) apiary. This facility is located at the Innovation Africa campus of the University of Pretoria in Hatfield, Pretoria, Gauteng Province, South Africa, and supports a number of long-term studies on honey bees. Naturally distributed throughout sub-Saharan Africa (Ruttner, 1988, Hepburn et al., 1998, Pirk, 2020), the distribution of this subspecies in South Africa specifically extends over much of the country except the Cape region, which is occupied instead by the country's other subspecies, *Apis mellifera capensis* (Crewe et al., 1994; Ruttner, 1988).

Pollen and nectar foragers were used as they are easily detectable at hive entrances, are regularly flight active, experience the greatest variation in temperature in the external environment, and experience the greatest exposure to pesticides in the environment while foraging. Upon inspection, we deemed experimental hives free of any obvious signs of disease and of a large enough size to withstand continuous removal of small numbers of honey bees over a prolonged period of time. Forager collection was done on warm days (25–40 °C) when forager traffic was high (no sample collection was done on cold/overcast/rainy days as there was minimal forager activity). To ensure that only foragers and not guard bees were collected, the hive entrances were smoked to ensure all bees at the entrance retreated inside and then the entrances (and any other visible openings) were blocked using small sections of foam. After a period of 5–10 min, the returning foragers collecting around the inaccessible entrances were collected using aspirators which were lined with foam to eliminate injury.

Neonicotinoid exposure

Once collected from hive entrances, forager bees were transferred to Perspex hoarding cages (120 mm x 95 mm x 80 mm) with sliding panels on both sides, a perforated panel for ventilation on the bottom and two small windows on the front to accommodate the insertion of two 2 mL centrifuge (Eppendorf®) tubes used to administer the diet (Köhler et al., 2013). Each cage contained 30 bees, maintained for no more than 2 days before using them in flight mill experiments, as the effects of chronic exposure remained the same for this period of time (Blanken et al., 2015; Tosi et al., 2017). All the tested foragers remained alive throughout the experiment. Foragers used in control (CONT) experiments were provided with two 2 mL microcentrifuge tubes containing only sugar water solution (1:1 w/w sucrose and water) for a period of 24 h prior to testing.

Three commercially available neonicotinoid active ingredients were used in the sublethal exposure treatments, namely thiamethoxam (THX), clothianidin (CLO), and imidacloprid (IMI). As per standard practice, acetone was used as an organic solvent for the neonicotinoids, to make them more soluble in the diet, with the total amount of acetone present per treatment, including the control, amounting to less than 0.05% (Aliouane et al., 2009; Démares et al., 2016; El Hassani et al., 2008). Field-realistic doses and concentrations of neonicotinoids tend to vary widely across space and time (Pisa et al., 2015). Neonicotinoid concentrations in this study were considered comparable to realistic field doses and the chronic period of exposure was sufficient to allow for all honey bees to consume sufficient treated sugar water to illicit any potential observable effects (Démares et al., 2018). Foragers used in the neonicotinoid treated trials were also provided with two 2 ml Eppendorf tubes, both treated with the same sub-lethal dose of the relevant neonicotinoid (40 µL in 2 mL tube of 1:1 w/w sucrose and water, final concentration of 5 nM), for a period of 24 h before being tested. These doses are well below the LD50 = 4–5 ng/bee threshold (Démares et al., 2016; Godfray et al., 2014; Henry et al., 2104; Oliveira et al., 2014).

Flight efficiency

The flight ability of active foragers was tested using a standard flight mill (Naranjo, 1990) (Supplementary Figure 1). Four flight mills were set up in the same incubator (Memmert HCP 108, GmbH + Co. KG, Schwabach, Germany) (Supplementary Figure 2) and maintained at a constant temperature of 25, 30 or 35 °C. Lighting in the incubator was provided by two standard daylight fluorescent tube lamps and the incubator door was fitted with a transparent observation pane. Flight experiments were performed between 9:00 and 17:00 during the winter, and 8:00 and 18:00 in summer, based on seasonal light and temperature changes and observed activity levels of the hives.

Following the 24 h, four foragers were removed from the cage at a time and anaesthetised in a cooler box of ice (approximately 5 °C) for 2–3 min. Foragers were not completely immobilised, merely cooled until leg and wing movements were minimal and a pin could be applied without any glue interfering with the wings. Each honey bee was attached to the flight mill by gluing the dorsal side of the thorax to the end of an insect pin using hot melt adhesive (HMA) that had been allowed to cool until safe for application but still adhesive. The end of the pin was bent to provide a 2 mm portion of the pin perpendicular to the main length of the pin, providing sufficient area for attachment while ensuring that it did not hinder the bee's wing movements. Once attached to the pins, the pins were attached to the end of one arm of a flight mill so that the bee was oriented the right way up. In order to ensure the flight mill arm remained exactly level during the experiment and to account for the weight of the honey bee, foragers were weighed prior to the experiment and a counterweight of similar weight (maximum deviation of 2.0 mg; Blanken et al., 2015) was attached to an identical pin fitted to the opposing side of the flight mill. Foragers were allowed to rewarm and regain full activity during a 10 min acclimatisation period at the given temperature before flight mill recording commenced and flight was stimulated. Forager flight activity was recorded on the flight mill for a period of 1 hr.

Flight mills were spaced within the incubator to minimise any interaction effects between neighbouring test subjects, and to ensure no contact with surrounding walls. Trial runs using various surrounding images and patterns showed little influence on stimulating flight activity in foragers. Small plastic circles of various colours (yellow, green, red and white) distributed on the shelves of the incubator proved to be the most effective visual stimulation. The four flight mills each had a diameter of 28 cm and an associated revolution of 87.97 cm. The flight mills were all connected to a specially designed data acquisition system which registered each half rotation and the time taken, recorded with specially designed software (Makumbe et al., 2020). Minimum conditions exist that constitute a successful flight and any honey bees measuring below that will be discarded and deemed a non-flight. Following initial behavioural observations, for the purposes of this study we defined a flight as a period between two breaks. A break was defined as a period of 3 s or longer without a revolution. A successful flight was considered to be three or more consecutive revolutions.

Baseline flight data for the control and the three neonicotinoid treatments was first determined at a functional ambient temperature of 25 °C in order to eliminate the effects of temperature on flight efficiency (Blanken et al., 2015; Brodschneider et al., 2009; Harrison et al., 1996). Control and neonicotinoid trials were also conducted at 30 and 35 °C. The selected temperatures are within the range of those at which honey bee flight is possible, with honey bees able to remain in continuous free flight at high air temperatures up to at least 46 °C (Heinrich, 1980).

Bees that failed to exhibit flight during the flight mill test period were designated as non-fliers. The percentage of successful flights was recorded overall as well as per treatment, and was used as one of several variables to evaluate the influence of neonicotinoids on honey bees.

Morphological traits

Because the flight capability of a honey bee can also be influenced by a suite of morphometric data, we recorded wing measurements and bee body weight. Directly following flight tests, honey bee samples were stored at −5 °C for later evaluation of morphometric measurements. Forager wings were carefully removed and wing slides were prepared for each honey bee. Each set of wings was mounted in a drop of distilled water on a glass slide and sealed with clear nail varnish before being photographed using a transmission light microscope (Vickers Instrument, York, England) equipped with a Moticam (Motic®, Moticam 5.0 MP, China). Using the photographs, the dimensions of each wing were measured using ImageJ image processing and analysis software (version 1.48, US National Institute of Mental Health, Bethesda, Maryland, USA). The measured wing traits were the area, length and width (in mm) of the left front (L1), left back (L2), right front (R1), and right back (R2) wings of each bee. The fresh weight of each bee was measured to the nearest 0.001 g (Blanken et al., 2015) using an electronic weighing balance (Mettler Toledo AG64, Greifensee, Switzerland).

Data analyses

The overall percentage of non-flights vs. successful flights was determined, as well as the percentage of non-flights vs. successful flights for each treatment and temperature condition. The flight mill data from successful flights was used to determine various tethered flight parameters including the number of flights, total distance flown and average flight speed. Total distance (m) was calculated using the number of laps and distance covered per lap (calculated using the radius of the flight path covered in one revolution, $c = 2\pi r$). Average speed ($m.s^{-1}$) was calculated using total distance (m) divided by total time flown (s).

In addition to the area, width and length of all four wings, we created two additional variables based on the symmetry of the wings. The area of the left wing was subtracted from the area of the right wing; meaning if wing symmetry was negative, the left wing was bigger, and if it was positive, then the right wing was bigger. A zero value meant the wings were symmetrical. Early exploratory data analysis where each of these wing measurements and bee weight (predictor variables) were plotted against flight success (response variable), showed no differences. Because the wing measurements are all inherently collinear and dependent on each other in some way, we opted to use a principal component regression with a binomial distribution to test whether weight and wing dimensions influenced whether the bee flew or not. A principal component analysis (PCA) was used to include all wing measurement variables and bee weight to create a list of orthogonal non-linear principal components done in R using base library *"stats"* (R Core Team, 2013). The principal components are Eigenvalues. Only the first 13 principal components were then used in a binomial regression against the predictor variable of whether the bee flew or not also in the R programming language (R Core Team, 2013). The first 13 were used because they explained 100% of the deviation in the data. A well performing model with significant principal components is an indication that wing measurements and bee weight would influence a bee's ability or likelihood to fly.

A generalised linear model (GLM) in programme R (R Core Team, 2013) was used to test the effects of neonicotinoids and temperature on flight success, number of flights, flight distance and average flight speed. For flight success, a binomial GLM with a logit-link function was used with the response variable being whether the bee flew (1) or not (0) using all 451 available data points. Only points where the bee flew were used for the remainder of the models (i.e., where the response value was not zero). The number of flights and flight speed followed a gamma distribution, determined using the "fitdistrplus" library (Delignette-Muller & Dutang, 2015) in R (R Core Team, 2013). Flight distance was severely right-skewed and was subsequently log-transformed after which a Poisson distribution was used in GLM modelling. Eight (number of flights), four (flight distance) and four (speed) extreme outlier points were not used in these analyses because they all represented anomalous bees who made between 61 and 126 flights; flew further than 2.8 kms; and flew faster than 5.5 m.s^{-1}. The excluded outliers recorded extreme values, with frenzied flight activity for the full hour of recording. Best models were chosen using chi-squared tests between models and comparing Akaike's information criterion values for small sample sizes (AICc) (Anderson & Burnham, 2004). Model fit was assessed using a variety of plots of residuals to make sure the fitted models met the assumptions.

Results

Morphological traits

The 13 principal components from the PCA explained 100% of the variance in the data (Supplementary Table 1). In the binomial GLM using the principal components as predictor variables only PC 7 was statistically significant, though explaining only 6.8% of the overall data variation (AIC = 130.2, Null deviance = 127.54, $df = 91$; Residual deviance = 102.2, $df = 78$) (Supplementary Table 1). Body mass did not differ significantly across the four treatments, at the three different temperatures suggesting it did not influence flight success (Supplementary Table 2). Therefore, we were confident that whether bees flew or not, it was not coincidentally influenced by the sample of bees we used to test the effects of neonicotinoids on bees.

Flight performance

We conducted flight mill runs on a total of 360 foragers across the three ambient temperatures and four dietary treatments. Of those foragers, 169 were designated as non-fliers and 191 were recorded to have performed successful flights. Overall, flight success was 53%. However, when evaluated per treatment the flight success was far lower in the neonicotinoid treatments than in the control. The binomial GLM determining whether a bee flew or not ($n = 451$) was only influenced by the treatment (log-ratio $\chi^2 = 53.4$, df = 3, $p < 0.001$) and not by temperature (Figure 1). The probability of flying was highest if a bee was in the control group (0.72; 95% CI = 0.64,0.78), followed by THX (0.46; 95% CI = 0.37,0.55), and CLO (0.41; 95% CI = 0.32,0.0.51), and lastly the probability of a bee flying was lowest for bees dosed with IMI (0.27; 95% CI = 0.19,0.37) (Figure 2).

When superficially examining the raw data there was a slight increase in the number of flights at 30 °C as compared to 25 °C and 35 °C (Supplementary Table 3). Both THX and IMI showed a decrease in number of flights with an increase in temperature, while CLO exhibited a noticeable increase at 30 °C as compared to 25 °C and 35 °C. The number of flights under control conditions did not vary significantly between treatments and temperatures. The best model for the number of flights included only neonicotinoid treatment as a predictor (AICc = 1378.3; ΔAICc = 0.00; df= 5; weight = 0.538) (Table 1).

The distance flown by the honey bees across the four treatments and three temperatures also varied greatly and showed no definitive trend (Supplementary Table 3). Similarly, the GLM following a Poisson distribution flight distance was not influenced by treatment, temperature or the interaction term and the null model was better than any of the other models with the various combinations of predictors (AICc = 678.8; ΔAICc = 0.00, df =1, Weight = 0.0701 vs AICc = 702.2; ΔAICc= 14.36, df = 8, Weight = 0.001 for the full model). The second ranked model had a ΔAICc value of 2.03 meaning it was not meaningfully different but not close enough

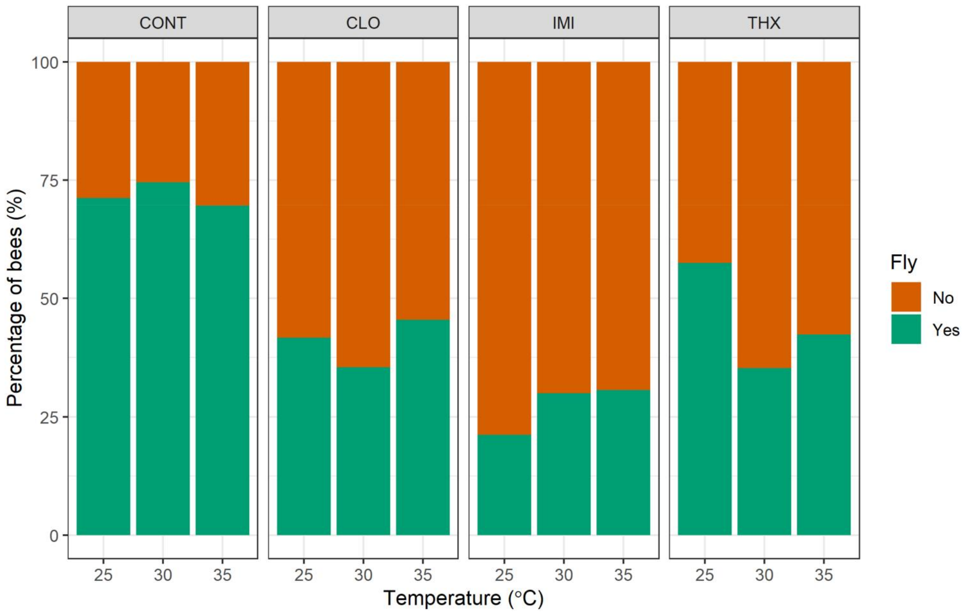

Figure 1. Summary plot showing the percentage of bees used in the experiment that flew (Yes) or not (No) for the control (CONT) and the three neonicotinoid treatments clothianidin (CLO), imidacloprid (IMI), and thiamethoxam (THX), under the three temperature conditions (25 °C; 30 °C, 35 °C).

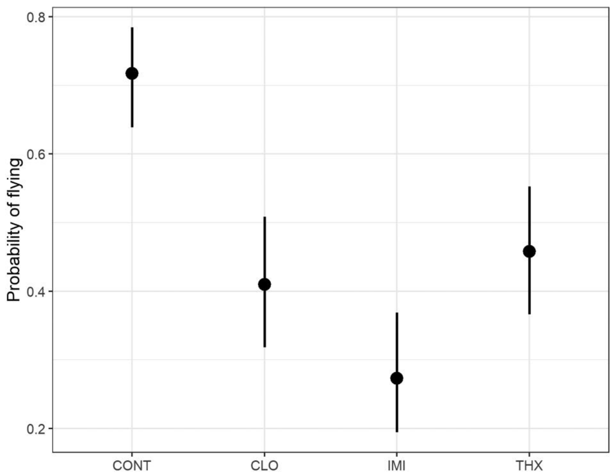

Figure 2. Model effects of binomial generalised linear model showing estimated mean and 95% confidence intervals of the probability of honey bees flying for the control (CONT) and the three neonicotinoid treatments clothianidin (CLO), imidacloprid (IMI) and thiamethoxam (THX).

Table 1. Generalised linear modelling to test the effects of neonicotinoid treatments (treat), temperature (temp) and their interacting effects (temp:treat) on the number of flights, flight distance and flight speed.

Model	*df*	logLik	AICc	ΔAICc	Weight
Number of flights					
Treat	5	−683.964	1378.3	0.00	0.538
Temp + treat	6	−683.679	1379.8	1.57	0.246
Intercept	2	−688.615	1381.3	3.03	0.118
Temp	3	−688.452	1383.0	4.77	0.049
Temp + Treat + temp:treat	9	−682.032	1383.1	4.83	0.048
Flight distance					
Intercept	1	−342.915	678.8	0.00	0.701
Temp	2	−342.908	689.9	2.03	0.255
Treat	4	−342.891	694.0	6.13	0.033
Temp + treat	5	−342.887	696.1	8.22	0.011
Temp + treat + temp:treat	8	−342.742	702.2	14.36	0.001
Flight speed					
Temp + treat + temp:treat	9	−194.630	408.3	0.00	0.710
Intercept	2	−204.199	412.5	4.21	0.087
Treat	5	−201.138	412.6	4.35	0.081
Temp	3	−203.348	412.8	4.57	0.072
Temp + treat	6	−200.534	413.5	5.27	0.051

df: degrees of freedom; logLik: Log Likelihood; AICc: Akaike's Information Criterion for small sample sizes; ΔAICc: the AICc difference between the current model and the most parsimonious model; Weight: Akaike weight, representing the relative support a model has from the data compared to the other models in the set.

to the null model to be considered a useful model (Anderson & Burnham, 2004).

Under control conditions, the distance flown differed minimally across the three temperatures. THX and IMI exhibited notably further distances flown at 35 °C and CLO showed similar distances flown across the three temperatures.

The best (lowest ΔAICc value) GLM following a gamma distribution for flight speed was the full model that included temperature, treatment and their interaction term (Table 1; AICc = 408.3; ΔAICc = 0.00; df= 9; weight = 0.710), with the second ranked model ΔAICc value increasing by 4.21 indicating a meaningful difference between the two models. Average flight speed was the highest for CLO at 25 °C (2,446 ± 2,028) (when temperature is assumed not to influence flight) and highest for CONT at both 30 °C (1,439 ± 1,437) and 35 °C (1,527 ± 1,188) (Supplementary Table 3).

However, the Chi-squared test indicated that none of the predictors were significant in the full model (Supplementary Table 4). From a biological perspective, the effects plot (Figure 3) indicates that flight speed increased with an increase in ambient temperature, with limited variation. The neonicotinoids IMI and THX showed a similar trend of increasing flight speed with increasing temperature whereas CLO exhibited a decrease (Figure 3).

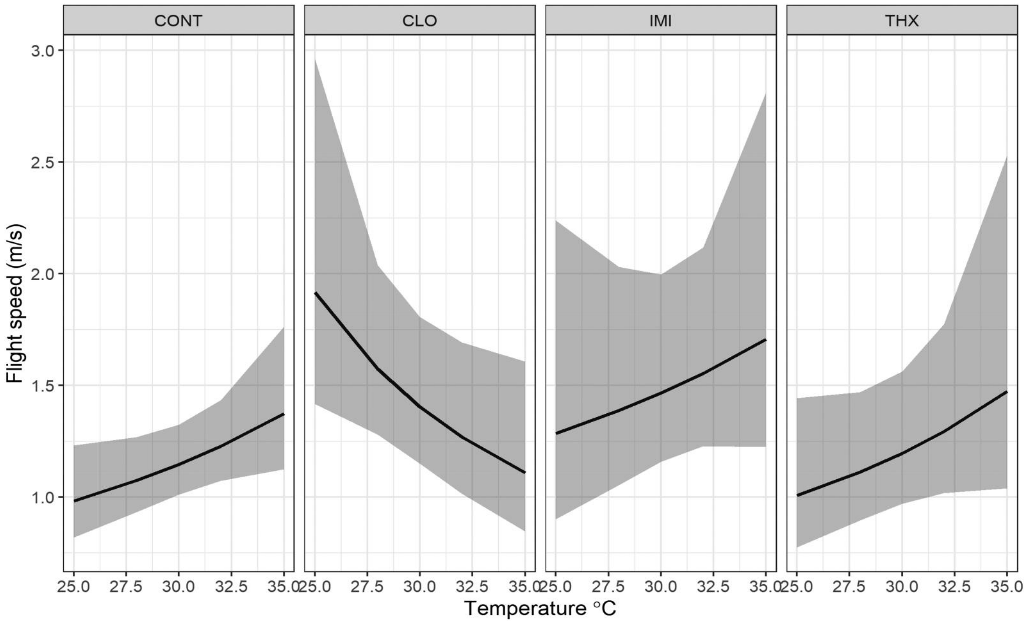

Figure 3. Model effects of gamma distributed GLM showing the estimated mean and 95% confidence intervals of average flight speed (m/s) for the control (CONT)and the three neonicotinoid treatments clothianidin (CLO), imidacloprid (IMI), and thiamethoxam (THX), under the three temperature conditions (25°C; 30 °C, 35 °C).

Discussion

When exposed to IMI, THX and CLO, under increasing ambient temperature conditions, *A. m. scutellata* exhibited increased variation in the number of flights, flight duration and flight speed, with a significant difference in flight success among treatments. Similar to Tosi et al. (2017), our bees were allowed to feed chronically on treated sucrose solutions for 24 h but rather than multiple concentrations of one neonicotinoid.

Initial flight success or failure was influenced only by treatment and the probability of a bee flying successfully was far higher in the control, with the three treatments having far lower flight success and IMI having the lowest. As far more bees did not fly at all under the neonicotinoid treated conditions as compared to the control, the lowered sample size increased the variation across these three treatments and likely contributed to the more varied results. While chronic sub-lethal THX has been shown to impair flight capacity in honey bees (Tosi et al., 2017), and IMI exacerbates the effect of *Varroa* infestation on flight capacity (Blanken et al., 2015) our study demonstrates a failure to initiate flight at all following chronic THX, IMI and CLO exposure, under all three temperature conditions. Whereas sublethal short-term THX exposure may have elicited an excitatory flight affect, chronic sub-lethal THX exposure tends to elicit a depressive long-term effect (Tosi et al., 2017; Ma et al., 2019). One of the neonicotinoids, THX, has been shown to affect thorax temperatures. Thorax temperature in *A.m. scutellata* was affected by THX exposure for at least 24 h if not longer, likely as a result of changes in thoracic muscle function (Tosi et al., 2016).

The number of flights initiated by the bees was influenced by treatment as well as the interaction between treatment and temperature. Under control conditions only a slight increase in flight number at 30 °C compared to 25 °C and 35 °C; THX and IMI decreased in flight number as temperature increased; while CLO noticeably increased at 30 °C as compared to 25 °C and 35 °C. Chronic THX exposure significantly decreased honey bee flight duration (−54%) (Tosi et al., 2017).

Flight distances were not notably influenced by either temperature, treatment or interaction terms. Descriptive statistics did show more of a variation in the THX and IMI treatments across the three temperature conditions, with far higher flight distances at the highest temperature (35 °C). Tosi et al., 2017 noted that chronic THX exposure resulted in a decrease in flight distance (−56%), citing the depressive effect of chronic exposure as a possible explanation. However, our results indicated that the number of flights was not significantly influenced by any of the predictor variables.

Apis mellifera subspecies in the Netherlands exhibited reduced flight distances and flight durations only when exposed to field-realistic, chronic sub-lethal doses of IMI in conjunction with high loads of the *Varroa destructor* mite (Blanken et al., 2015). The sub-lethal effects of IMI alone were not sufficient to elicit a notable change in the flight parameters (Blanken et al., 2015). The South African subspecies experiences a lowered load of *Varroa destructor* mite which likely results in a similarly limited influence on the flight parameters.

Flight speed was affected by temperature, treatment and their interaction term but the chi-squared test indicated none of the predictors were significant in the full model. While average flight speed may not have been significantly affected by temperature or treatment, the results still have broader biologically relevant implications. Under control conditions, average flight speed increased with an increase in ambient temperature conditions. Ambient temperatures around 25 °C do not affect flight, as oxygen consumption and metabolic rate are comparatively lower than at other ambient temperatures (Blanken et al., 2015; Brodschneider et al., 2009; Harrison et al., 1996; Hrassnigg & Crailshiem, 1999). There was far greater variation in flight speed under neonicotinoid conditions as opposed to the control. Chronic THX exposure slightly decreased honey bee average velocity (−7%) (Tosi et al., 2017). Exposure to neonicotinoids such as THX can induce short-term hyperactivity which can in turn lead to long-term muscular exhaustion and lowered energetic availability (Derecka et al., 2013; Tosi et al., 2017) which could account for the greater variation in flight speed observed among the neonicotinoid treated honey bees. Both IMI and THX chronic exposure has been shown to elicit immediate excitation and hyperactivity followed by decreased activity and responsiveness in bees (Gill & Raine, 2014; Suchail et al., 2001; Tosi et al., 2017), an effect that could explain the fact that these two neonicotinoids had similar progressive effects on flight number, distance and speed across the three temperatures. CLO on the other hand, while still a neonicotinoid which acts on nicotine acetylcholine receptors (nAChR), targets nAChR subtypes that differ from those of THX and may have slightly different effects (Simon-Delso et al., 2015; Tosi et al., 2017), possibly accounting for the contrasting effects of CLO on the flight parameters.

Wing dimensions and body weight did not appear to have any effect on whether a bee flew successfully or not. The fact that only 6.8% of the overall variation in the data was explained by the model is most likely an effect of sample size and does not hold any biological significance While morphometric measurements did not have any bearing on flight success in this study, the difference in results between our study and similar studies on other subspecies (*A. m. mellifera* x *A. m. carnica* x *A. m. buckfast,* subspecies hybrid, Blanken et al., 2015; *A. m. ligustica,* Tosi et al., 2016) may be in part due to physical differences among these subspecies. For example, mass-specific metabolism of active, flying *A. m. scutellata* is higher than in honey bees of European subspecies due to the larger thorax-specific capacity and higher flight muscle oxidative capacity leading to a difference in flight capacity and metabolism among honey bee subspecies (Harrison & Hall, 1993; Hepburn et al., 1999; Kovac et al., 2014).

Considering that PC 7 only explained 6.8% of the overall variation in the data, this is most likely an effect of sample size and does not hold any biological significance

Conclusions

Our study uses flight success and associated flight parameters as an indication of flight muscle function and by extension an indicator of honey bee capacity to thermoregulate both physiologically *via* thermogenesis and behaviourally *via* wing fanning and water foraging. Neonicotinoid induced alterations to various flight parameters and reduced flight success may not only affect the nectar and pollen foraging capacity of a colony and the subsequent nutritional diversity of foraged pollen (Tison et al., 2016; Tosi et al., 2016), but also the efficacy of individual and colony level behavioural and physiological thermoregulation. While these findings are a step in the right direction for field applicable results, further investigation into these conditions and parameters on actual pesticide treated crops rather than laboratory-based dosing would provide further valuable information (Thompson et al., 2016). This study serves as a strong base from which future studies under more extreme weather conditions would yield valuable information on how the *A.m. scutellata* honey bee subspecies will continue to adapt and be affected by accelerating changes to global and local climatic conditions, especially in the context of an ever-changing South African agricultural landscape. Future research could be conducted over a longer study period, looking at all seasons, on multiple sites and across the extensive *A.m. scutellata* distribution range. Additionally, future investigations can be conducted on the efficacy of current honey bee thermoregulatory mechanisms in response to future extreme weather conditions over an extended period.

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Data availability statement

Data will be made available on Research Gate.

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