

1 **Pesticide exposure affects flight dynamics and reduces flight endurance in bumblebees**

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14 **Running Title**

15 Pesticide impairs bee flight performance

16

17 **Summary Statement**

18 Acute neonicotinoid exposure impaired flight endurance and affected velocity of *Bombus terrestris* workers,  
19 which may dramatically reduce colony foraging potential and pollination provision in pesticide applied  
20 landscapes.

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22

## 23 **Abstract**

24 The emergence of agricultural land use change creates a number of challenges that insect pollinators, such as  
25 eusocial bees, must overcome. Resultant fragmentation and loss of suitable foraging habitats, combined with  
26 pesticide exposure, may increase demands on foraging, specifically the ability to reach resources under such  
27 stress. Understanding the effect that pesticides have on flight performance is therefore vital if we are to assess  
28 colony success in these changing landscapes. Neonicotinoids are one of the most widely used classes of pesticide  
29 across the globe, and exposure to bees has been associated with reduced foraging efficiency and homing ability.  
30 One explanation for these effects could be that elements of flight are being affected, but apart from a couple of  
31 studies on the honeybee, this has scarcely been tested. Here we used flight mills to investigate how exposure to  
32 a field realistic (10ppb) acute dose of imidacloprid affected flight performance of a wild insect pollinator - the  
33 bumblebee, *Bombus terrestris audax*. Intriguingly, initial observations showed exposed workers flew at a  
34 significantly higher velocity over the first  $\frac{3}{4}$  km of flight. This apparent hyperactivity, however, may have a cost  
35 as exposed workers showed reduced flight distance and duration to around a third of what control workers were  
36 capable of achieving. Given that bumblebees are central place foragers, impairment to flight endurance could  
37 translate to a decline in potential forage area, decreasing the abundance, diversity and nutritional quality of  
38 available food, whilst potentially diminishing pollination service capabilities.

39

## 40 **Introduction**

41 The extent to which insects move across landscapes has significant implications for human welfare.  
42 Highly mobile species can potentially lead to detrimental insect pest outbreaks (Mazzi and Dorn, 2012; Sharov  
43 and Liebhold, 1998), invasions (Myers et al., 2000; Renault et al., 2018) or the spread of vector borne diseases  
44 (Dujardin et al., 2008; Estrada-Peña et al., 2014; Githeko et al., 2000; Rogers and Packer, 1993). Yet insect  
45 movement can also underpin beneficial ecosystem service provision. For example, the majority of angiosperms,  
46 including around  $\frac{3}{4}$  of our crop species, are to some degree reliant upon the extensive movement of foraging  
47 insect pollinators (Gill et al., 2016; Kleijn et al., 2015; Klein et al., 2007; Ollerton et al., 2011). It is therefore  
48 important we understand which, and to what extent, stressors can impact on insect pollinator flight  
49 performance if we are to mitigate threats to a global pollination service valued at >€150bn annually (Benaets et  
50 al., 2017; Fischer et al., 2014; Gallai et al., 2009; Gill and Raine, 2014; Stanley et al., 2015; Wolf et al., 2014).

51 The emergence of intensive agriculture can cause loss and fragmentation of suitable foraging habitats,  
52 leading to resources becoming increasingly sparse and isolated within an insect's foraging range (Didham et al.,  
53 1996; Hadley and Betts, 2012; Steffan-Dewenter and Tschardtke, 1999; Tschardtke and Brandl, 2004; Zurbuchen  
54 et al., 2010). This may pose a considerable challenge for eusocial bees, which are characterised as central place  
55 foragers by having a fixed nest site. Workers must undertake return foraging trips from this set nest location,  
56 and consequently any habitat discontinuity may require workers to fly longer distances to find and bring back  
57 resources, such as pollen and nectar (Goulson et al., 2008; Jha and Kremen, 2013; Pelletier and McNeil, 2003;  
58 Schmid-Hempel and Schmid-Hempel, 1998). Hence any stressor lowering individual worker flight ability could

59 translate to negative colony level impacts (Gill et al., 2012), with implications for the crucial ecosystem services  
60 they provide (Delaplane and Mayer, 2000; Garibaldi et al., 2013; Greenleaf and Kremen, 2006; Potts et al., 2010;  
61 Winfree et al., 2008).

62 Insecticides are commonly applied in agricultural landscapes as a pest management strategy  
63 (Fernandez-Cornejo and Vialou, 2014; Ramankutty et al., 2018), with neonicotinoids being one of the most  
64 widely used classes worldwide (Simon-Delso et al., 2015). However, neonicotinoids have been implicated as a  
65 threat to eusocial bees (Gill et al., 2012; Goulson, 2013; Lundin et al., 2015; Tsvetkov et al., 2017; Whitehorn et  
66 al., 2012; Woodcock et al., 2017). Foraging eusocial bees are known to be frequently exposed to neonicotinoids  
67 in treated landscapes (Botías et al., 2015; Botías et al., 2017; David et al., 2016; Mitchell et al., 2017; Rolke et al.,  
68 2016), and controlled exposure experiments have demonstrated both impaired homing ability (Fischer et al.,  
69 2014; Stanley et al., 2016) and foraging efficiency of workers, including longer foraging trips and reduced rate of  
70 pollen collection (Feltham et al., 2014; Gill and Raine, 2014; Stanley and Raine, 2016). A possible explanation for  
71 these reported impairments is that certain aspects of foraging flight dynamics, such as endurance and speed,  
72 are affected by neonicotinoid exposure. However, to date only two studies (both using tethered honeybees)  
73 have specifically tested this and have reported mixed findings. One study found acute neonicotinoid exposure  
74 increased flight endurance, with the opposite effect shown following chronic exposure (Tosi et al., 2017). The  
75 other study detected no effect of chronic exposure on flight performance unless it was provided to individuals  
76 in combination with the parasitic varroa mite (Blanken et al., 2015). Hence, further investigation is needed to  
77 understand the generality of the effects of exposure on bee flight, whilst also: i) ensuring that a concentration  
78 within the field realistic range is used; ii) gaining a more in-depth analysis of the dynamics of flight during testing;  
79 iii) investigating a representative species of wild bee, given there can be differential responses to pesticide  
80 exposure between insect pollinator species (Cresswell et al., 2012; Heard et al., 2017; Rundlöf et al., 2015); and  
81 iv) considering variation in worker body size, given this can modulate flight capability, can be associated with  
82 variation in foraging behaviours within bumblebee colonies (Goulson et al., 2002; Spaethe and Weidenmuller,  
83 2002), and size-specific energetic demands show a non-linear relationship (Greenleaf et al., 2007; Kaufmann et  
84 al., 2013).

85 We investigated the effect of acute oral neonicotinoid exposure on different aspects of bumblebee  
86 (*Bombus terrestris audax*) flight performance using a controlled tethered flight mill setup. For this study we  
87 exposed individual workers to the neonicotinoid imidacloprid at a concentration of 10ppb as it is: i) a widely  
88 used insecticide across the globe with a growing market in many regions (Casida, 2018; Cressey, 2017; Domenica  
89 et al., 2017; Mitchell et al., 2017; Zhang, 2018); ii) a concentration that can be found inside social bee colonies,  
90 on return foraging workers, and in the pollen and nectar of individual flowers (Blacquièrre et al., 2012; Botías et  
91 al., 2016; Cresswell, 2011; Dively and Kamel, 2012; Goulson, 2013; Hladik et al., 2016); iii) known to impair  
92 foraging performance after exposure (Godfray et al., 2014; Pisa et al., 2017); and iv) a neonicotinoid under  
93 current scrutiny by policy makers and regulators (Cressey, 2017), resulting in a recent EU ban from agricultural  
94 use outside of closed greenhouses. Here, we tested the propensity of individual bees to fly, followed by

95 measures of their flight distance and duration, the dynamics of velocity over the course of the flight test, and  
96 investigated how neonicotinoid exposure interacted with worker body size on these performance measures.

97

## 98 **Methods**

### 99 ***Bee husbandry***

100 Three bumblebee *Bombus terrestris audax* colonies, containing a queen and between 130-150 workers,  
101 were supplied by a commercial company (Agralan Ltd). Each colony was delivered in a separately housed plastic  
102 nest box (29 x 22.5 x 13 cm) and kept in a controlled environment room (25°C) under red light. From the point  
103 of arrival, colonies were provisioned with 4g of pollen daily and supplied with ad libitum 10/90% sucrose/water  
104 solution via a connected reservoir. A 10% sucrose concentration falls within the range of many flower species  
105 (Pierre et al., 1999; Pyke and Waser, 1981), but our primary justification for using this concentration was to  
106 ensure individual workers were motivated to feed to satiation when provided a higher concentration of sucrose  
107 solution during the acute exposure setup, whilst ensuring that provisioned colonies did not suffer from  
108 dehydration or starvation.

109

### 110 ***Flight Mill Setup and Bee Tethering***

111 Six flight mills were set up in a separate adjoining room under the same environmental conditions as  
112 the housing room (constant 25°C temperature), but with the option to switch between red (Philips TLD 58W Red  
113 1SL/25; mean 660 nm) and white light (Philips TLD 58W 840). Flight mills were adapted from a previous design  
114 (Smith and Jones, 2012), consisting of a revolving brass wire, with a magnet hanging from one end designed to  
115 attach to a metal tag glued to the bee's thorax through magnetic attraction (Fig. 1). The revolving brass wire was  
116 suspended over a central Delrin rod by the repulsive forces of two magnets, preventing friction during arm  
117 rotation to allow fluid motion. The Delrin rod was positioned vertically (90° perpendicular) on a horizontally flat  
118 triangular Perspex base. A digital Hall-effect sensor placed on one side of the mill detected each complete  
119 revolution by the passage of a neodymium magnet (Fig. 1A) and sent an impulse to a Raspberry Pi computer  
120 (model B) via a copper wire connector. From here a Python script recorded the time (in seconds) between each  
121 impulse, with each revolution defined as a 'circuit' from hereon.

122 When the colonies arrived, we randomly selected 110 workers per colony (total=330) and under red  
123 light attached a circular galvanised iron tag (diameter = 2 mm, thickness = 0.4 mm) to the thorax of each worker  
124 using super glue (Fig. 2), allowing each individual to be tethered to the hanging flight mill magnet (Fig. 1B,D). We  
125 were confident that tag mass would not cause any significant impairment to bee flight performance, as mean  
126 ( $\pm$ s.e.m) tag mass was  $18 \pm 0.3$  mg (calculated from weighing 30 tags), equating to just 7.5% of the mean worker  
127 wet mass of all individuals tested in this study ( $240 \pm 5$  mg). Indeed, bumblebees are capable of carrying > 50%  
128 of their own body mass in nectar alone when foraging (Brian, 1954). Each tag was placed at the centre of the  
129 thorax, with the tag leading edge touching the back of the first thoracic stripe (Fig. 2). This placement ensured

130 no impediment of wing movement when attached to the flight mill. The meticulous nature of tagging each  
131 bumblebee meant that we scored tag positions as 1 = ideal, 2 = unideal or 3 = poor (Fig. 2), with scores 1 and 2  
132 being considered acceptable to experimentally test but score 3 being excluded from further use. In total, 74  
133 workers per colony were tested (total = 222).

134

### 135 **Pesticide Preparation**

136 A 10ppb imidacloprid working solution was produced to supply the acute pesticide exposure to  
137 worker individuals. A previously made stock solution of 1ppt imidacloprid dissolved in acetone was used which  
138 was stored in a freezer wrapped in aluminium foil to prevent light degradation (Soliman, 2012). Aliquots of the  
139 stock solution were diluted by addition of a 50% sucrose solution to create the working 10ppb *pesticide*  
140 treatment solution. The control solution was made by adding the respective volume of acetone to the 50%  
141 sucrose solution to produce a 10ppb acetone *control* working solution.

142

### 143 **Experimental Procedure**

144 Pilot studies were first conducted in September 2015 and March 2017 to trial and verify the  
145 experimental setup and procedures, with the main experimental study conducted in April 2017. The main  
146 experimental testing started 12 hours after tagging was completed, and testing took place over an 8-day period.  
147 Workers were tested in bouts, with 5-6 bouts undertaken per day. Six workers were sampled per bout (one per  
148 flight mill), consisting of two workers sampled per colony, with one worker randomly assigned to the *treatment*  
149 and the other to the *control*. This ensured that all three colonies and both treatment groups were represented  
150 equally in each bout and over the totality of the experiment (total: n = 37 bees per treatment per colony; n =  
151 111 *control* & 111 *pesticide*). Once removed from the colony, each worker was directly transferred to a separate  
152 transparent horizontally laid Perspex tube (length = 150mm, ID = 19mm). The tube had a rubber bung at each  
153 end creating a holding compartment for the bee, with individuals left to acclimatise for a resting period of 3  
154 minutes. After this resting period, one of the bungs was replaced with cotton wool lightly soaked in the *control*  
155 or *pesticide* treatment sucrose solution (Fig. 1C). This already piloted method ensured that 94.1% of workers in  
156 our main experiment fed. We made the assumption that spiking the sucrose solution with the neonicotinoid  
157 would not deter feeding if given no other option, as supported by pilot observations and previous studies (Arce  
158 et al., 2017; Gill et al., 2012; Kessler et al., 2015). Our pilot study also indicated that workers took a mean ( $\pm$ s.e.m)  
159 duration of  $50 \pm 13$  seconds to commence feeding (defined as prolonged proboscis extension on to the cotton  
160 wool) and fed for a mean ( $\pm$ s.e.m) duration of  $213 \pm 24$  seconds before stopping, with subsequent feeds being  
161 rare, sporadic, and short (<10 seconds). Workers could access the provisioned sucrose-soaked cotton wool for  
162 10 minutes, after which the cotton wool was removed and original bung replaced, followed by a 5-minute resting  
163 period inside the tube. Whilst this protocol meant that we could not determine the precise dosage of  
164 imidacloprid consumed by each worker, it did allow workers to feed to satiation which is a state likely to occur

165 in the field during foraging bouts, and importantly allows consumption volume to vary proportionately to  
166 individual worker size (Free and Butler, 1959; Goulson et al., 2002)

167         The workers that fed were then removed carefully using tweezers and tethered to the flight mill. The  
168 5.9% of workers that did not feed were immediately frozen (-20°C) and weighed along with all other bees after  
169 all flight tests had been completed. All of this was carried out under red light conditions, but once workers were  
170 tethered to the mills the room was switched to white light. Each mill had a separate height-adjustable stand  
171 which was erected once the bee was tethered and used to hold the worker in place (Fig. 1D). Prior to initiating  
172 the flight test, workers were held in place for a period of 10 minutes for two primary reasons: i) pilot observations  
173 demonstrated that some bees were initially irritated by attachment to the mill which discouraged flight, but that  
174 a 10 min acclimatisation period allowed irritation to subside; ii) a balance was sought between giving workers  
175 time to metabolise the neonicotinoid and preventing de-motivation to fly by having them separated from their  
176 natal colony for too long. Studies have shown that honeybees metabolise imidacloprid and other neonicotinoids  
177 quickly, with a 100µg kg<sup>-1</sup> dose of imidacloprid showing the greatest levels of presence in the thorax and  
178 abdomen after just 20 minutes from ingestion (Suchail et al., 2004), and >50% of a 100µg kg<sup>-1</sup> dose of acetamiprid  
179 being metabolised in less than 30 minutes (Brunet et al., 2005). In our study, a total of 25 mins passed from  
180 starting the feeding trial to starting the flight test, which we are therefore confident represents enough time for  
181 absorption and metabolism of some of the imidacloprid consumed.

182         Immediately after the 10-minute acclimatisation period, the support stand was removed quickly from  
183 beneath the bee in order to stimulate flight. Prior to removal, the stand was rotated to ensure the worker had  
184 a forward-facing orientation. Stand removal caused loss of tarsal contact with the stand surface, which can  
185 trigger flight as evidenced in our pilot and other previous studies (Blanken et al., 2015; Brodschneider et al.,  
186 2009; Tosi et al., 2017). However, if the worker did not initially start flying the flat side of the stand was used to  
187 gently tap the legs in order to generate a sharp loss of tarsal contact. Up to three taps were allowed in this first  
188 flight attempt, with individuals being removed from the flight test if no flight was initiated.

189         Workers that successfully flew in the first attempt were monitored for any subsequent flight stoppages.  
190 Each stop was noted down as 'a strike', and each worker was permitted five strikes before their flight test was  
191 terminated. Immediately following a strike, the individual would be held in the stand to ensure tarsal contact  
192 for a 20 second rest period before removal of the stand again. Therefore, in the subsequent data analysis, flight  
193 stoppages were identified as circuits with a duration >20 seconds. After a strike, workers were only permitted  
194 one tap of the legs in attempt to trigger flight, otherwise their flight test was terminated. Any stoppages that  
195 occurred on the first circuit were discounted as genuine stoppages, as this was deemed an acclimatisation circuit  
196 for bees to familiarise themselves with the experimental setup. All workers were given the opportunity to fly for  
197 up to 60 minutes, including all stops, after which the flight test was terminated. Each individual worker was  
198 allowed a maximum of five 'strikes', essentially allowing each bee five chances to continue its flight until the 60-  
199 minute end point. We felt this provided a better representation of field conditions and a more realistic prediction  
200 of foraging distances, as foraging bees do not fly continuously during foraging bouts but will stop at flowers  
201 periodically to feed and rest (Woodgate et al., 2016). Additionally, it allows individuals to acclimatise to the

202 conditions of the flight mill and decreases the possibility of excluding individuals from testing that are initially  
203 demotivated to fly due to the experimental set up.

204         Following each flight test, workers were placed in separate labelled tubes and frozen (-20°C). After  
205 completion of the whole experiment, for each individual worker we measured: i) wet body mass (including the  
206 attached metal tag); and ii) intertegula span (ITS) taken using a digital calliper (Workzone 150mm), with the  
207 mean of three repeated measurements being used. For our data analysis, ITS was taken as a proxy for worker  
208 body size (Cane, 1987; Greenleaf et al., 2007). This is more appropriate than considering worker wet mass, as  
209 wet mass will vary according to both the volume of sucrose solution consumed and the duration of flight, as  
210 individuals gain mass through feeding and lose it through energy metabolism during flight.

211

## 212 **Data Cleaning**

213         Frequency distribution plots revealed a spike in the number of workers that terminated the flight test  
214 before completing 100m (118 circuits; Fig. S1A). Workers that did not fly over this threshold distance were  
215 excluded from the endurance and velocity analysis as a precautionary measure to discount individuals whose  
216 flight mill performance is not representative of actual flight capacity. For each worker flying beyond the 100m  
217 threshold, we calculated the following: i) total distance flown during the flight test, by taking the total number  
218 of circuits flown multiplied by the circuit circumference (0.848m); ii) total duration of the flight test, by summing  
219 all circuit interval times; and iii) velocity of each circuit, by taking the circuit circumference and dividing it by the  
220 respective circuit interval time. We took a simple calculation for mean velocity, calculated as the total distance  
221 flown divided by the total duration flown, and maximum velocity was taken from the circuit showing the highest  
222 velocity attained across the flight test.

223         The velocity calculations for each individual flight test were carried out on cleaned data in which the  
224 following circuits were excluded from the analysis: i) first five circuits of the first flight attempt; ii) first five  
225 circuits directly following a strike; iii) the circuit directly preceding a strike circuit. It was noted from pilot  
226 observations and the main study that removal of the support stand or tapping of the legs would often stimulate  
227 strikingly high velocities. It is likely this behaviour is a reaction to stimulatory stress, so we felt actions i) and ii)  
228 were justified as a precautionary measure to ensure we only considered circuits representative of normal  
229 continuous flight. Similarly, in justification of action iii), the minimal rotational resistance of the mill means that  
230 when a worker stops flying it does not equate to an abrupt stop, but the brass arm continues to rotate and slows  
231 gradually.

232

## 233 **Data and Statistical Analysis**

234         When considering total duration flown it was noted that the data was bimodally distributed (Fig. S1B),  
235 therefore we converted the results to a binary response variable categorised as having or having not flown >2000  
236 seconds, with this duration value decided on as it fell at the bottom of the bimodal concave.



237 Statistical analyses were conducted using the 'lme4' (Bates et al., 2015) package in R v3.2.0 (R Core  
238 Team, 2015), with summary statistics generated using the package 'psych' (Revelle, 2015) and results reported  
239 using the package 'lmerTest' (Kuznetsova et al., 2015). A linear model was used to compare variation in *ITS* (body  
240 size) between treatments, with *treatment* (*control* or *pesticide*) as the only fixed effect. For all other analyses,  
241 mixed effects models (fitted by maximum likelihood) were initially used with *colony* included as a random effect.  
242 Unless otherwise stated, fixed effects in each analysis included *treatment* (*control* or *pesticide*), *ITS* and the  
243 associated interaction term. Where response variables were binary (propensity to feed, propensity to fly, flight  
244 over 100m, flight longer than 2000 seconds) the data were analysed using a GLMM function under a binomial  
245 family distribution, with an LMM function used for all other responses (feeding time, total distance flown, mean  
246 velocity, maximum velocity). However, where the random effect of *colony* was found to explain none of the  
247 variance in the data, it was removed from the model to simplify, and the model reverted to either a GLM or LM  
248 instead (the type of model used is indicated with each result). To examine whether an unideal (score 2) tag  
249 fitting inhibited flight or impeded movement, we compared the propensity to fly (GLM) and distance flown (LM)  
250 between tag ratings for both *treatment* groups separately. Here, the fixed effects were *tag rating* (score of  
251 1=ideal or 2=unideal), *ITS* and the interaction between the two. Flight velocity over time (considering flight over  
252 the first 900 circuits) was analysed using an LMM function with the random effect structure nesting individual  
253 bee ID within circuit to account for individual repeated measures over time, and fixed effects including  
254 *treatment*, *ITS*, *circuit*, and the interaction term between *treatment* and *circuit*. The model suffered from high  
255 Eigen values and had trouble converging when considering all 900 repeated measures, therefore to enhance  
256 model fit and convergence we scaled the *circuit* variable and considered the average velocity of every 50<sup>th</sup> circuit  
257 (i.e. each bee had a mean per circuit velocity for circuits 1 to 50, 51 to 100, 101 to 150 and so on) resulting in 18  
258 repeated measures. In all cases, model residuals were plotted to confirm the data met the parametric  
259 assumptions of the tests used. Where appropriate, normality tests were used to reveal distributions of the data,  
260 and those which appeared non-normal were suitably transformed, with details of these found in Appendix 1.

261

## 262 **Results**

### 263 **Feeding behaviour**

264 We found no significant effect of treatment on the propensity to feed ( $n=9$  *control* & 4 *pesticide* workers  
265 did not feed; GLM:  $z=1.29$ ,  $p=0.20$ ). In concordance with our pilot observations, we found that any feeds  
266 following the first were sporadic and short, suggesting workers fed to relative satiety on their first feed.  
267 Therefore, we used the length of first feeding time as a reliable proxy for total feeding time. Of the 209 workers  
268 that fed, the mean ( $\pm$ s.e.m) time spent feeding was  $138 \pm 9.0$  seconds ( $n=102$ ) and  $127.2 \pm 7.8$  seconds ( $n=107$ )  
269 for *control* and *pesticide* workers respectively, with no significant difference between treatments (LMM:  $t=-0.77$ ,  
270  $p=0.44$ ; Fig S2). For the flight mill testing, however, we decided to include only those workers that fed for >60  
271 seconds (*control* = 86, *pesticide* = 94, total = 180; Table 1), because: i) visualisation of the plotted feeding times  
272 suggested initial feeds <60 seconds were relative outliers (Fig S2); and ii) we wanted to increase the likelihood  
273 that each worker had fed to satiation. We found that whilst body size was not a significant predictor of feeding



274 time (LMM:  $t=1.37$ ,  $p=0.172$ ), the propensity to feed increased with increasing body size (GLM:  $z=2.643$ ,  
275  $p=0.008$ ).

276

## 277 **Flight behaviour**

278 The flight data from 140 of the 180 bees tested on the flight mill were analysed (Table 1), as four workers  
279 were not considered due to flight mill technical difficulties, and 36 not considered because unideal (score 2) tag  
280 application appeared to affect aspects of flight performance (please see below for justification).

281

### 282 *i) Effect of tag fitting*

283 The propensity of workers to fly was not significantly affected by tag rating (GLM: *control*;  $z=-0.98$ ,  
284  $p=0.33$ ; *pesticide*;  $z=-0.04$ ,  $p=0.97$ ), although it is interesting that a higher percentage of tag rating 1 (ideal fitting)  
285 workers flew compared to tag rating 2 (unideal fitting) workers in both the *control* (71% vs 61%) and *pesticide*  
286 (76% vs 72%) groups. When considering total distance flown, however, tag rating 2 workers flew a significantly  
287 shorter mean distance compared to tag rating 1 bees in the *control* group (640 vs 1436 m respectively; LM:  $t=-$   
288 2.189,  $p=0.033$ ), with a similar, although non-significant, trend observed in the *pesticide* group (191 vs 415 m;  
289 LM:  $t=-1.643$ ,  $p=0.11$ ). Furthermore, we saw similar patterns in other flight metrics with tag rating 2 bees  
290 showing lower total duration flown (*control* = 1114 vs 2132 secs; *pesticide* = 272 vs 553 secs) and slower mean  
291 velocity (*control* = 0.562 vs 0.657 m/s; *pesticide* = 0.618 vs 0.744 m/s). It was therefore decided to exclude all 36  
292 tag rated 2 workers (*control* = 18, *pesticide* = 18; Table 1) from our analyses, to avoid any artefact results.

293

### 294 *ii) Initial flight behaviour*

295 Flight was initiated by 103 workers, comprising 71% of *control* ( $n=47$  of 66) and 76% of *pesticide* workers  
296 ( $n=56$  of 74), revealing a similar propensity to fly between treatments (GLM:  $z=0.50$ ,  $p=0.62$ , Table S1). Body size  
297 was found to be a significant predictor of propensity to fly, with the likelihood of flying increasing with *ITS* (GLM:  
298  $z=2.163$ ,  $p=0.031$ ; Table S1). This translated to an estimated probability of *control* workers initiating flight of  
299 0.49, 0.77 and 0.92 for workers with a 4mm, 5mm, and 6mm *ITS* respectively, with a similar pattern observed  
300 for *pesticide* workers (Fig. 3). *Pesticide* compared to *control* workers demonstrated a significantly higher  
301 termination of the flight test within the 100m threshold at a proportion of 0.43 ( $n=24$  of 56) vs 0.26 ( $n=12$  of 47;  
302 GLM:  $z=-2.115$ ,  $p=0.035$ ; Table S1) respectively. For instance, a *control* worker with 5mm *ITS* had an estimated  
303 proportion of 0.81 chance of flying >100m, compared to just 0.62 for a *pesticide* worker of the same *ITS*. We  
304 further found that larger *ITS* significantly increased the probability of flying >100m (GLM:  $z=2.318$ ,  $p=0.020$ ),  
305 with no clear significant difference in this relationship between treatments (GLM: *treatment\*ITS*:  $z=1.86$ ,  $p=0.06$ )

306

### 307 *iii) Flight endurance & velocity*

308           Inspection of the 67 bees that flew >100m showed an uneven *ITS* distribution between treatments,  
309 with a significant bias of larger *pesticide* workers (mean *ITS* of  $4.83 \pm 0.05$  mm vs  $4.99 \pm 0.04$  mm for *control* vs  
310 *pesticide* workers respectively; LM:  $t=2.382$ ,  $p=0.020$ ). We therefore took a conservative approach and ran two  
311 separate analyses on: i) the full dataset including all 67 bees (*control* = 35, *pesticide* = 32); and ii) a subset of the  
312 data (*control* = 26, *pesticide* = 27) in which we attempted to normalise the worker *ITS* distribution by removing  
313 the smallest 10% ( $n = 6$  *control* &  $1$  *pesticide*) and largest 10% ( $n = 3$  *control* &  $4$  *pesticide*) of workers; resulting  
314 in no significant difference in worker *ITS* between treatments ( $4.86 \pm 0.03$  mm vs  $4.94 \pm 0.03$  mm for *control* vs  
315 *pesticide* workers respectively; LM:  $t=1.77$ ,  $p=0.08$ ; Table 1). Normalising the dataset allowed us to better meet  
316 the assumptions of our implemented linear models, therefore here we present the analysis using the data  
317 subset, and provide the results using the full dataset in the supplementary material (Fig. S3 & Table S2), which  
318 showed the same directional pattern on flight performance between treatments.

319           *Pesticide* workers flew a significantly lower mean ( $\pm$ s.e.m) total distance at just  $659.1 \pm 78.7$  m  
320 compared to  $1,833.9 \pm 207.6$  m for *control* (LMM:  $t=-5.618$ ,  $p<0.001$ ; Fig. 4A, Table S2). The effect of pesticide  
321 exposure on distance flown was mirrored in the effect on duration flown, with a mean ( $\pm$ s.e.m) flight duration  
322 of just  $822.0 \pm 90.8$  seconds for *pesticide* exposed workers being considerably shorter than  $2,852.2 \pm 234.4$   
323 seconds for *control* workers (Fig. 4B, Table S2). Visualisation of the durations flown across all workers (Fig. 4B)  
324 showed a striking difference between treatments, with a proportion of just 0.04 of *pesticide* workers flying  
325 >2000 seconds, whilst 0.81 of *control* workers surpassed this duration (GLMM:  $z=-4.016$ ,  $p<0.001$ , Table S2).  
326 Furthermore, a proportion of 0.65 of *control* workers flew for the full 60 minutes permitted, whereas critically  
327 not one *pesticide* exposed worker achieved this.

328           Interestingly, the effect of worker body size on distance flown appeared to differ between *pesticide* and  
329 *control* groups, as indicated by a significant *treatment\*ITS* interaction (LMM:  $t=-2.242$ ,  $p=0.029$ ; Fig. 4A, Table  
330 S2). Separate analysis of each treatment group showed that whilst increasing *ITS* resulted in significantly higher  
331 total distances for *control* workers (LMM:  $t=2.158$ ,  $p=0.041$ ), this relationship was not found for *pesticide*  
332 exposed workers (LMM:  $t=-1.03$ ,  $p=0.31$ ; Fig. 4A). The effect of *ITS* on total duration flown showed the same  
333 general trend as that found for distance (Fig. 4B, Table S2), however, the difference in effect between treatments  
334 was less strong (GLMM:  $z=-1.720$ ,  $p=0.085$ ). Separate analyses for each treatment group found no significant  
335 relationship between *ITS* and the proportion of bees flying >2000 seconds for both treatments (GLMM: *control*:  
336  $t=1.50$ ,  $p=0.13$ ; *pesticide*:  $t=-1.13$ ,  $p=0.26$ ).

337           When considering the velocity of individuals across the total flight period, we found *pesticide* exposed  
338 workers attained a significantly higher mean ( $\pm$  s.e.m) velocity of  $0.84 \pm 0.05$  m/s per worker compared to  $0.63$   
339  $\pm 0.04$  m/s for *controls* (LMM:  $t=2.954$ ,  $p=0.005$ ; Fig. 4C, Table S2). Looking at maximum velocity, whilst we found  
340 no significant difference between treatments (LMM:  $t=1.58$ ,  $p=0.12$ ; Fig. 4D, Table S2), it was intriguing that  
341 *pesticide* exposed workers were again faster on average (mean  $\pm$  s.e.m =  $1.52 \pm 0.06$  m/s vs.  $1.34 \pm 0.09$  m/s).  
342 This consistent pattern motivated us to examine where these differences in velocity may stem from during flight.  
343 Visualisation of velocity over time suggested that *pesticide* workers maintained a higher velocity compared to  
344 *controls* during the initial phase (the earlier circuits) of the flight test (see Fig. 5). It also showed a sharp decline

345 in velocity around 900 circuits (760m) as a large proportion of *pesticide* workers terminated flight. Therefore,  
346 focusing on the first 900 circuits, we reveal that *pesticide* workers did fly significantly faster compared to *control*  
347 workers (LMM:  $t=3.459$ ,  $p=0.001$ ; Table S3), with this difference between treatments maintained over these  
348 circuits (*treatment\*circuit* interaction:  $t=1.862$ ,  $p=0.07$ ). Neither mean or maximum velocity was significantly  
349 predicted by worker *ITS* (LMM:  $t=1.60$ ,  $p=0.12$  &  $t=1.00$ ,  $p=0.32$  respectively; Fig. 4C,D, Table S2), and there  
350 appeared to be no effect of *ITS* on velocity over the first 900 circuits (LMM:  $t=0.50$ ,  $p=0.62$ , Table S3).

351

## 352 Discussion

353 Despite the importance of bumblebee foraging ability in providing a key pollination service (Garibaldi  
354 et al., 2013; Kleczkowski et al., 2017; Stanley et al., 2015), this study, to our knowledge, is the first to test how a  
355 specific stressor directly affects the properties of flight in bumblebees. Our findings demonstrate that acute  
356 exposure to the neonicotinoid pesticide, imidacloprid, was sufficient to significantly impact overall flight  
357 endurance, reducing flight distance and duration to around a third of what *control* workers were able to achieve.  
358 Whilst initially both *control* and *pesticide* exposed workers were equally motivated to fly initially, *pesticide*  
359 exposed workers showed a higher probability of terminating flight before the end of the 60-minute flight test,  
360 which was even evident within the first 100m. Intriguingly, *pesticide* workers exhibited a higher mean velocity  
361 compared to *control* workers, which was underpinned by faster flight speeds over the course of the first  $\frac{3}{4}$  km,  
362 both during and after which we observed a considerable proportion of pesticide workers terminating their flight.  
363 Furthermore, our results suggest that pesticide exposure may negate the capability of larger workers to fly  
364 longer distances than their smaller sister workers.

365 The degree of impact that an acute neonicotinoid exposure had on reducing bumblebee worker flight  
366 endurance observed in our study did come as a surprise, as a previous honeybee study showed acute exposure  
367 to thiamethoxam increased flight endurance. One possible explanation for these contrasting results is the  
368 structural differences between thiamethoxam and imidacloprid compounds, which bind to different sites on  
369 nicotinic acetylcholine receptors (nAChRs) with variable affinity (Iwasa et al., 2004; Kayser et al., 2004; Marletto  
370 et al., 2003; Wiesner and Kayser, 2000). Indeed, studies have previously shown bumblebees to be less sensitive  
371 to thiamethoxam compared to imidacloprid when considering effects on brood production and food  
372 consumption (Heard et al., 2017; Laycock et al., 2014). That said, the only other flight mill study to test  
373 neonicotinoid effects on honeybee flight capacity also used imidacloprid (as in our study), yet found no effect  
374 on workers free from infection with the *Varroa* mite (Blanken et al., 2015). Therefore, this reinforces the view  
375 that responses to pesticide exposure can vary considerably even between closely related genera. Indeed, both  
376 lab (Cresswell et al., 2012) and field (Rundlöf et al., 2015) studies have highlighted differences in neonicotinoid  
377 effects between honeybees and bumblebees, with large interspecific differences in the toxicity of pesticides  
378 over time (Heard et al., 2017). Together this emphasises the growing appreciation that reported effects on  
379 honeybees cannot always be extrapolated to other wild bees, and highlights the danger of using honeybees as

380 lone indicator species for insect pollinator responses to pesticides (Gill et al., 2016; Heard et al., 2017; Raine and  
381 Gill, 2015).

382 Our flight tests suggest that imidacloprid exposed bumblebee workers experienced a rapid de-  
383 motivation to fly as the test progressed and/or tired quickly leading to premature physical exhaustion. Our study  
384 was not designed specifically to test these two non-mutually exclusive explanations, however given that only 4%  
385 of *pesticide* exposed workers flew >2000 seconds (*control* = 81%) and that not one individual completed the 60-  
386 minute test (*control* = 65%), our findings suggest that physical ability may have been affected, which could then  
387 have subsequently led to demotivation. We found no difference in initial motivation to fly and in fact *pesticide*  
388 exposed workers flew faster than *control* workers, inferring that immediate motor function was not impaired  
389 *per se*, but instead flight stamina was reduced. Neonicotinoids have been implicated in affecting honeybee  
390 energy metabolism (Derecka et al., 2013), and imidacloprid has been shown to reduce mitochondrial activity,  
391 impairing respiratory processes and causing rapid mitochondrial depolarization in neurons of bumblebees and  
392 honeybees (Moffat et al., 2015; Nicodemo et al., 2014). Given the high energy expenditure required during flight,  
393 a reduction in mitochondrial functioning and the consequent inhibition of ATP production in flight muscles could  
394 lead to rapid muscle exhaustion, which might explain our findings of significantly reduced endurance.  
395 Imidacloprid can also induce the down-regulation of genes involved in sugar metabolising pathways in honeybee  
396 larvae (Derecka et al., 2013), which if true for bee adults could seriously impact flight performance that requires  
397 muscles to function at high glycolytic rates (Staples and Suarez, 1997). It is interesting to note that buzz  
398 pollination by bumblebees, whereby the creation of resonant vibrations from the flight muscles dislodges pollen  
399 from anthers (Morgan et al., 2016), has also been reported to be impaired by neonicotinoid exposure  
400 (Whitehorn et al., 2017).

401 Neonicotinoids are agonists of insect nicotinic acetylcholine receptors (Déglise et al., 2002) and can  
402 acutely increase neuronal activity (Matsuda et al., 2001; Moffat et al., 2016). A resultant effect of this may be  
403 individual hyperactivity of specific tasks, which could explain our observations of higher velocity in exposed  
404 workers during the initial phase of the flight test, and has been previously suggested to underpin neonicotinoid  
405 effects on honeybee flight and locomotor activity (Lambin et al., 2001; Suchail et al., 2001; Tosi et al., 2017).  
406 Bumblebee colony level exposure to imidacloprid has also been shown to lead to a higher number of workers  
407 going out to forage (Gill et al., 2012), a pattern that could be an adaptive response to filling a foraging deficit,  
408 but could also be down to possible maladaptive hyperactive behaviour. Additionally, our study suggests a  
409 potential cost to hyperactivity, as exposed workers terminated flight prematurely which may have been due to  
410 increased energy expenditure during the initial phase leading to faster muscle fatigue and energy depletion, but  
411 further testing would be needed to understand this. In sum, our results highlight the importance of looking at  
412 the pattern of flight dynamics, rather than experimental end-points, to better understand the mechanisms  
413 behind how neonicotinoids act and their temporal effects (Suchail et al., 2001; Wen and Scott, 1997).

414 Bumblebees have been reported to exhibit a certain degree of alloethism, whereby worker body size  
415 can determine divisions in colony tasks (Goulson et al., 2002; Herrmann et al., 2018; Peat et al., 2005). Larger  
416 workers of a colony are considered more likely to become committed foragers (Jandt and Dornhaus, 2009;

417 Spaethe and Weidenmuller, 2002), and there have been reports of foraging rate, distance and efficiency (nectar  
418 collected per unit time) increasing with body size (Goulson et al., 2002; Greenleaf et al., 2007; Jandt and  
419 Dornhaus, 2009; Kapustjanskij et al., 2007; Spaethe and Weidenmuller, 2002; Worden et al., 2005). Whilst our  
420 study found no clear relationship with flight velocity and body size, we did find that both the propensity to fly  
421 and total flight distance were positively related in *control* workers, which might provide a mechanistic  
422 explanation as to why foragers tend to be the larger colony workers. Critically, however, we found no such  
423 significant relationships in *pesticide* exposed workers, suggesting that the negative effect of neonicotinoid  
424 exposure on flight actually increased in magnitude as workers increased in body size. Intriguingly, a previous  
425 study showed that neonicotinoid induced impairment to spatial learning behaviour in bumblebees appeared to  
426 be exhibited more highly in the largest colony workers (Samuelson et al., 2016). Together these findings raise  
427 the question as to whether larger bumblebees are more susceptible to pesticide effects. With pesticide exposure  
428 seemingly counteracting the increased flight performance with body size, the production of larger bees could  
429 be seen as wasted energetic investment for the colony. Further investigation is required to look at this, however,  
430 as whilst the interactive effect of pesticide and body size was detected in the subset of workers analysed, this  
431 effect seemed to be lost when considering the full dataset; a discrepancy that may stem from biases in worker  
432 size between treatments as a consequence of the flight trial filtering process.

433 Bumblebee foraging ranges are difficult to accurately measure, and further knowledge of this important  
434 behaviour is critical for predicting colony success and pollination services in changing landscapes. Our flight mill  
435 setup showed *control* workers to fly a mean total distance of 1.8km, which appears to sensibly conform to other  
436 estimates of bumblebee foraging ranges. Estimated foraging ranges using different techniques including  
437 harmonic radar (Osborne et al., 1999), mark-recapture (Kreyer et al., 2004; Osborne et al., 2008), and use of  
438 microsatellite genetic markers (Darvill et al., 2004) for *Bombus terrestris* vary from 0.34 to 2.2km. As bumblebees  
439 are central place foragers, foraging trips require not only reaching a resource, but also returning to the nest site  
440 after collection of food or other resources. The minimum round-trip flight distances associated with the above  
441 foraging ranges would therefore span from around 0.68km to 4.4km. Given that our measures fall in the middle  
442 of these estimates, we are confident that our flight mill test setup can provide us with meaningful insights into  
443 the effects of stress on flight capabilities that can occur in the field. With imidacloprid exposure reducing total  
444 flight distance by nearly 1.2km on average, this corresponds to a 64% reduction in comparison to the *control*,  
445 which would lead to a notable 87% decline in the total foraging area accessible to a colony (using the colony as  
446 the epicentre). Pesticide exposure will therefore place increased stress on bumblebee colonies, with foragers  
447 potentially being unable to reach resources they previously could, or unable to return to the nest following  
448 exposure feeding on contaminated flowers. Not only would this reduce the abundance, diversity, and nutritional  
449 quality of food available to a colony, but could also reduce the pollination service the colony is able to provide  
450 (Blanken et al., 2015; Tosi et al., 2017; Van der Sluijs et al., 2013). Looking at the effects of chronic exposure  
451 would provide further insights, as bees in the wild would likely be exposed to treated or contaminated flowering  
452 plants throughout the season (Simon-Delso et al., 2015; Stanley et al., 2013; Tison et al., 2016).

453

454 **Contributions**

455 RJG conceived the project; DK analysed the data; HC, IP, ARR & SG developed the experimental setup; HC  
456 performed the experiment; DK, HC and RJG wrote the manuscript.

457

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461

462 **Competing Interests**

463 No competing interests declared

464

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469

470 **Experimental Animals**

471 All procedures involving experimental animals were performed in compliance with local animal welfare laws,  
472 guidelines and policies.

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762 **Figure legends**

763 **Figure 1. Flight mill setup and associated experimental procedures.** Panels show A) flight mill used in the study  
764 (the 'height adjustable screws' ensured the mill could be horizontal with an attachable bubble level used to  
765 ensure this); B) tethering of an individual worker bumblebee to the flight mill magnet; C) feeding procedure in  
766 which workers were placed in bunged tubes with one end consisting of cotton wool lightly soaked in 50% sucrose  
767 solution ( $\pm$  10ppb imidacloprid); D) support stands used to hold workers prior to flight tests and following a stop  
768 in flight.

769

770 **Figure 2. Example of the ideal positioning of a metal tag (tag score 1) on the thorax of a *Bombus terrestris***  
771 ***audax* bumblebee worker.** If the tag positioning was unideal (tag score 2) the metal tag would overlap the yellow  
772 circle but remain inside the blue circle. If positioning was unacceptable (tag score 3) it would overlap the blue  
773 circle.

774

775 **Figure 3. Logistic regression plot showing the effect of body size (*ITS*) on the propensity to fly.** All workers from  
776 both treatments were pooled (n=140) and could either have initiated flight (= 1) or refused to fly (= 0; Table 1 –  
777 filter step 4).

778

779 **Figure 4. Scatterplot showing key flight performance indicators of endurance (distance flown in meters (A);**  
780 **duration flown in seconds (B)) and average and maximum velocity in meters per second (C-D) against worker**  
781 **body size (*ITS*) for both the *control* (red) and *pesticide* treated (blue) groups.** Data plotted is for the subset of  
782 bees with normalised *ITS* between treatments (number of workers = 26 *control*; 27 *pesticide*), and linear fitted  
783 lines with 95% confidence intervals are estimates of the mixed effects models.

784

785 **Figure 5. Mean velocity (m/s) flown by each treatment group (*control* = red, *pesticide* = blue) plotted for each**  
786 **consecutive circuit for just the first 2500 circuits.** Numbers at the bottom of the graph refer to the number of  
787 bees still flying on the corresponding circuit, and the data plotted is for the subset of bees with normalised *ITS*  
788 between treatments (starting number of workers = 26 *control*; 27 *pesticide*). Vertical line represents the first  
789 900 circuits used in the analysis for initial individual velocity, and the associated error per mean circuit velocity  
790 is not shown.

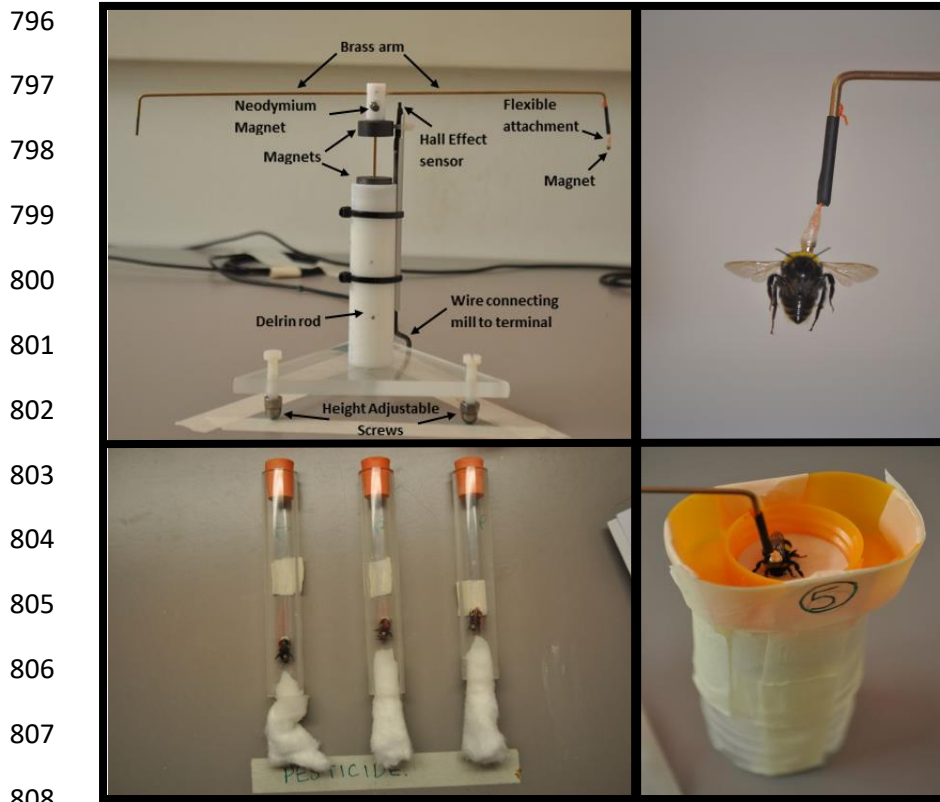
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795 **Figures**



809 **Fig 1**

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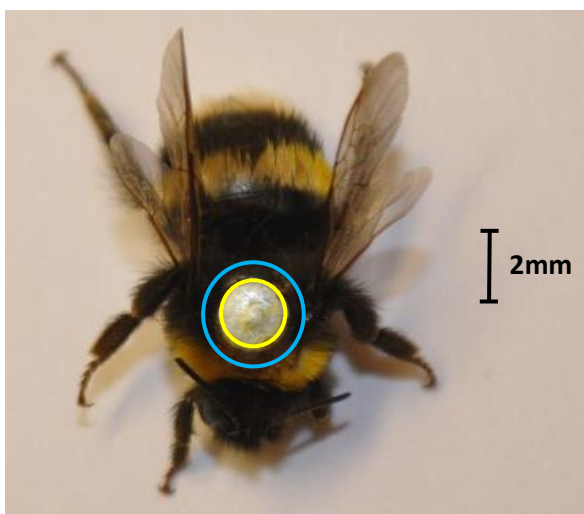
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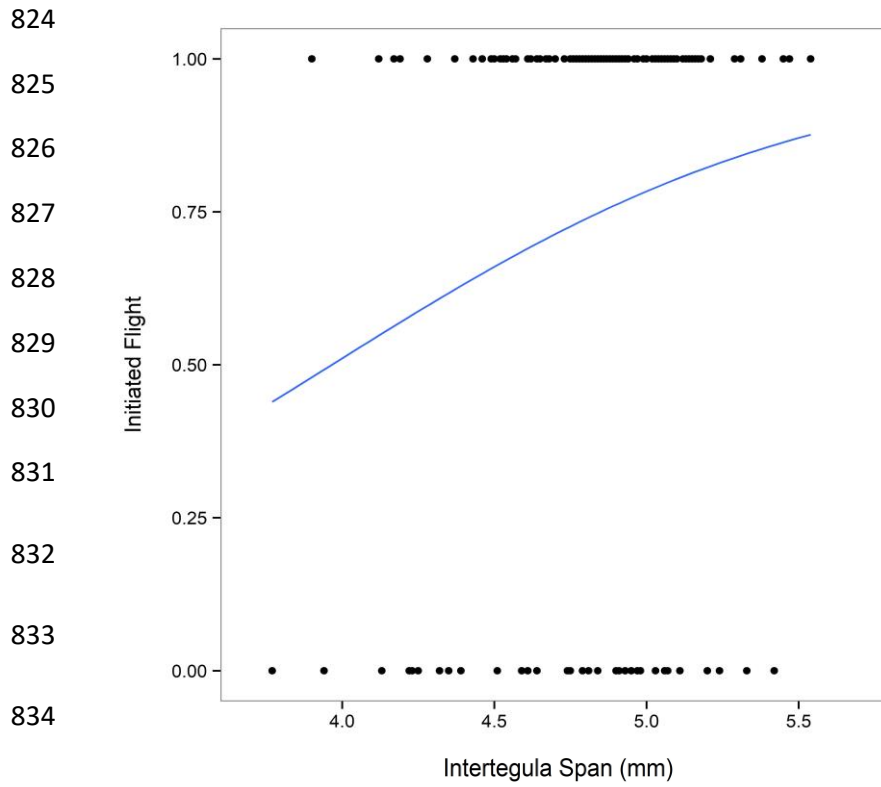
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822 **Fig 2**

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836 **Fig 3**

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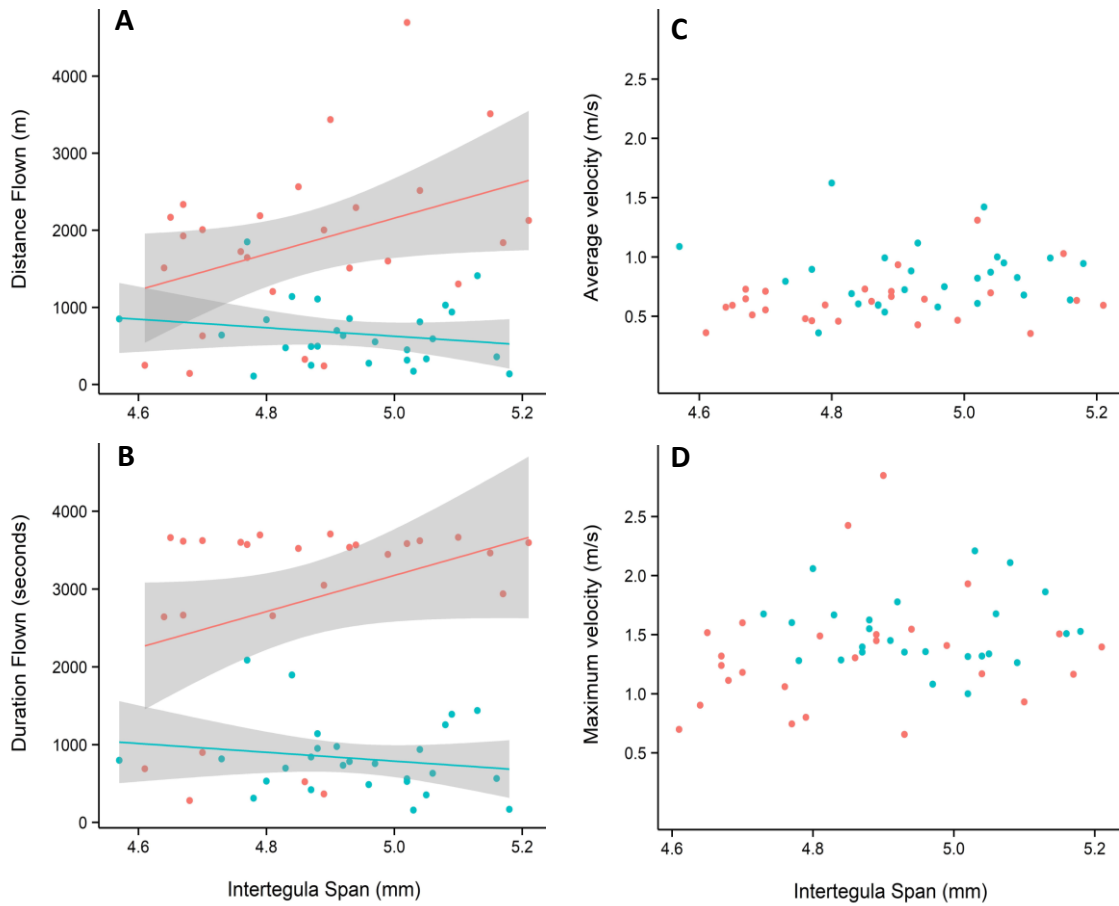
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866 **Fig 4**

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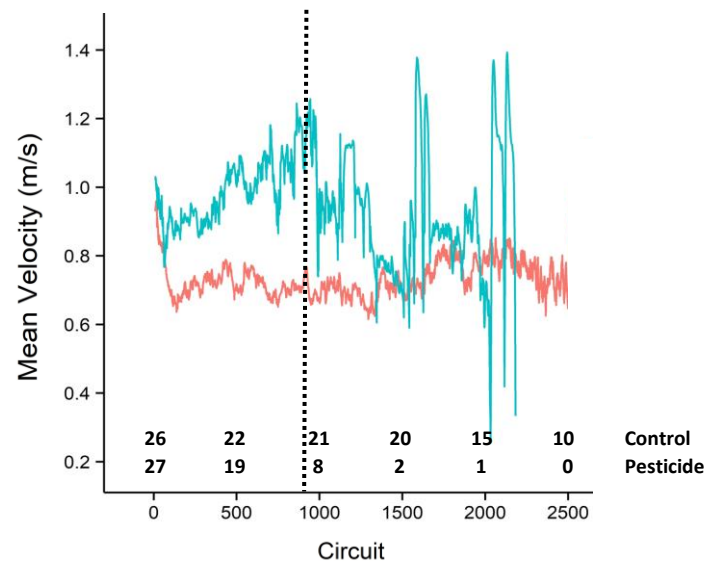
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879 **Fig 5**



880 **Tables**

	<b>Control</b>	<b>Pesticide</b>	<b>Total</b>
Total bees at start	111	111	222
Did not feed	9	4	13
Fed	102	107	209
<b>Filter Step 1</b>	<b>102</b>	<b>107</b>	<b>209</b>
Fed <60 seconds	16	13	29
Fed >60 seconds	86	94	180
<b>Filter Step 2</b>	<b>86</b>	<b>94</b>	<b>180</b>
Technical difficulties	2	2	4
Used in flight mill study	84	92	176
<b>Filter Step 3</b>	<b>84</b>	<b>92</b>	<b>176</b>
Tag Rating 2	18	18	36
Tag Rating 1	66	74	140
<b>Filter Step 4</b>	<b>66</b>	<b>74</b>	<b>140</b>
Did not fly	19	18	37
Flew	47	56	103
<b>Filter Step 5</b>	<b>47</b>	<b>56</b>	<b>103</b>
Flew <100m	12	24	36
Flew >100m	35	32	67
<b>Filter Step 6</b>	<b>35</b>	<b>32</b>	<b>67</b>
Removed top and bottom 10% sized individuals	9	5	14
Remaining bees for final analysis	26	27	53
<b>Filter Step 7</b>	<b>26</b>	<b>27</b>	<b>53</b>

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882 **Table 1. An overview of the filter steps used when cleaning the data for analysis of flight performance,**  
883 **outlining the number of workers removed from each treatment at each stage.**

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## Appendix 1

### **Pesticide exposure alters flight dynamics and reduces flight endurance in bumblebees**

Daniel Kenna\*, Hazel Cooley\*, Ilaria Pretelli, Ana Ramos Rodrigues, Steve D. Gill & Richard J. Gill

#### **Statistical Analysis**

In all cases, model residuals were plotted to confirm the data met the parametric assumptions of the tests used, and model fit was assessed and optimised where possible. Where appropriate, normality tests were used to reveal distributions of the data, and those which appeared non-normal were suitably transformed as outlined below:

#### Feeding time

The feeding time response variable was box-cox transformed (Venables and Ripley, 2002) to the optimal exponent.

#### Effect of tag fitting on flight behaviour

The effect of tag fitting on flight behaviour was examined separately for both treatments. The effect of tag fitting on total distance flown was analysed using a linear model, with the response variable being square root transformed when considering the *pesticide* treatment, but being left untransformed when considering the *control* treatment.

#### Average and maximum velocity

Prior to maximum velocity analysis, one outlier was identified in the *pesticide* treatment group with a maximum velocity of 14 m/s. This value was around 12 m/s higher than any other individual, and being considered a reaction to stimulation as opposed to normal flight behaviour this value was removed from further analysis. When considering the full dataset of 67 bees (Table 1 – filter step 6), the average velocity and maximum velocity values were square root and cube root transformed respectively. When only considering the subset of 53 bees (Table 1 – filter step 7, both the average velocity and maximum velocity values had to be square root transformed.

#### Total distance flown

For both the full dataset and the subset analysis, the total flight distance response variable was square root transformed.

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