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**Exposure to pollen-bound pesticide mixtures induces longer-lived but less efficient  
honey bees**

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20

## 21 **Abstract**

22 Due to the widespread use of pesticides and their persistence in the environment, non-target organisms  
23 are chronically exposed to mixtures of toxic residues. Fungicides, herbicides and insecticides are all  
24 found at low doses in the diet of pollinators such as honey bees, but due to the lack of data on the  
25 toxicological effects of these mixtures, determining their risk is difficult to assess. We therefore  
26 developed a study combining the identification of common pollen-bound pesticide mixtures associated  
27 with poor colony development and tested their effects on bee behavior and physiology. We exposed  
28 bees to the identified pesticide mixtures during the first days of their adult life, a crucial period for  
29 physiological development. Using optic bee counters we recorded the behavior of bees throughout  
30 their lives and identified two pesticide mixtures that delay the onset of foraging and slow-down  
31 foraging activity. Furthermore, one of these mixtures hampers pollen foraging. As bee longevity is  
32 strongly influenced by the time spent foraging, bees exposed to these pesticide mixtures outlived  
33 control bees. Physiological analysis revealed that perturbations of the energetic metabolism preceded  
34 the altered behavior. In conclusion, we found that early-life exposure to low doses of pesticide  
35 mixtures can have long-term effects that translate into longer-lived but slower and less efficient bees.  
36 These surprising findings contrast with the commonly reported increase in bee mortality upon  
37 pesticide exposure, and demonstrate that exposure that may seem harmless (*e.g.*, very low doses,  
38 pesticides not intended to kill insects) can have undesirable effects on non-target organisms.

39

## 40 **Highlights**

- 41 • Two mixtures of 4-5 fungicides and 1 insecticide perturbed bee energetic metabolism
- 42 • These physiological perturbations slowed down the bees' foraging activity
- 43 • Additionally, one of the mixtures hampered pollen foraging
- 44 • Exposure to the two mixtures resulted in longer-lived but less efficient bees

45

46 **Keywords**

47 Foraging, fungicides, honey bee, insecticides, pollen, survival.

48

## 50 1. Introduction

51 The widespread use of chemicals to control pests has permeated the biosphere (air, water and soil)  
52 with toxic residues, causing the exposure of non-target organisms to a large number of contaminants at  
53 relatively low doses (Barbash, 2003; Gavrilesco, 2005; Looser et al., 2000). However, the risk that  
54 multiple residues might pose to non-target species is difficult to assess due to the lack of data on the  
55 toxicological effects of mixtures and clear evidence of the actual exposure (Lydy et al., 2004). The  
56 diversity of residues to which organisms are exposed makes testing each possible exposure scenario  
57 unreasonable. The uncertainty of the actual risks involved fosters controversy between different  
58 sectors of our society; policy makers, farmers, consumers, scientists and environmentalists tend to  
59 have contrasting views on the issue (Storck et al., 2017). The controversy is particularly evident  
60 regarding the contention of the involvement of pesticide residues in the health of insect pollinator  
61 populations (Suryanarayanan and Kleinman, 2013; Vogel, 2017).

62 One solution towards understanding the risks presented by mixtures is through pesticide monitoring  
63 programs that combine exposition with pollinator population dynamics, i.e. identifying common  
64 pesticide mixtures that cause adverse effects (Lydy et al., 2004). The challenges involved in such an  
65 approach include proper ways of linking exposure data to population dynamics, as well as  
66 implementing experimental designs that permit testing hypotheses. We therefore have developed a  
67 study combining the identification of field-relevant pesticide mixtures (associated with perturbations  
68 in bee colony development) with experimental testing of their effects on bee behavior and physiology.

69 The honey bee (*Apis mellifera*), as an important pollinator of crops worldwide, is commonly exposed  
70 to pesticide mixtures in agricultural areas (Poquet et al., 2016; Sanchez-Bayo and Goka, 2014). When  
71 visiting flowers, bees can encounter agricultural chemicals (e.g. insecticides, acaricides, fungicides  
72 and herbicides) and bring them back to the hive, where they are unintentionally stored in honey, bee  
73 bread (stored pollen) and wax. One of the primary routes of exposure is via residues in the pollen,  
74 which is the resource most commonly polluted due to its physical characteristics (i.e. highly sculptured  
75 cavities filled with a lipophilic pollen coat) (Mullin et al., 2010). Broad surveys of pollen pollutants in

76 Europe and North America report a high incidence of contamination in the pollen collected by bees,  
77 with 27 to 100 % of the samples contaminated by at least one pesticide (Bernal et al., 2010; Chauzat et  
78 al., 2006; Genersch et al., 2010; Mullin et al., 2010; Porrini et al., 2016). Despite the fragmentary  
79 information on bee pollen pollutants, a common picture emerges where pollen in agricultural areas is  
80 more often than not contaminated by more than one pesticide, with a high prevalence of fungicides.  
81 Pollen as the honey bee's main source of proteins, amino acids and fat, is consumed in high quantities  
82 during the first ten days of adult life (Winston, 1991). As a consequence, during this early-life period  
83 of intense pollen consumption, adult bees can be chronically exposed to mixtures of pollutants that are  
84 present in pollen. Even if the current level of exposure has raised international concerns about the role  
85 of pesticides in the severe colony losses (Hayes Jr et al., 2008; Nguyen et al., 2010; Potts et al., 2010),  
86 we have a limited understanding of the toxicity of pesticide mixtures that occur in the environment  
87 (Böhme et al., 2017).

88 Our approach to evaluate the effects of pesticide residues in pollen involved first analyzing the  
89 exposure of colonies located near agricultural areas, and choosing potentially hazardous mixtures  
90 based on the prevalence of compounds, their co-occurrence, their mode of action, and their potential  
91 toxicity to colonies (negative brood growth rate). Then, through manipulative experimentation, we  
92 tested the effects of pollen contaminated with these mixtures on bee flight activity and survival. Honey  
93 bees provide an interesting toxicological model as the activity of hundreds of marked individuals can  
94 be recorded throughout their lives using automatic monitoring systems at the entrance of the hive  
95 (Streit et al., 2003). We hypothesized that as observed with other bee stressors (i.e. parasites,  
96 malnutrition) bees exposed to pesticide mixtures in their pollen diet would start foraging earlier and  
97 die younger (Goblirsch et al., 2013; Schulz et al., 1998). However, contrary to our expectations, our  
98 data revealed a consequence of pesticide exposure never previously described: longer-lived but slower  
99 and less efficient bees. Accordingly, we evaluated the impact of pesticide mixtures on bee energetic  
100 metabolism and foraging performance, and simulated the observed effects on long-term colony  
101 dynamics.

102

## 103 2. Methods

### 104 2.1. Identification of potentially hazardous pesticide mixtures from field 105 surveys

106 Pesticide mixtures were selected based on the prevalence of compounds in the field, their mode of  
107 action, their potential toxicity, and their co-occurrence. For that purpose, we used the 2014 dataset  
108 from the French Observatory for Pesticide Residues in the Honeybee Diet, which included the pollen-  
109 bound pesticide residues and the growth dynamics of 25 colonies situated near crop fields. Colonies  
110 were distributed in 3 regions (Midi-Pyrénées, Provence-Alpes-Cotes d'Azur and Rhône-Alpes) and  
111 monitored between March and October 2014. Capped brood on all frames was estimated on a monthly  
112 basis by visual inspection by trained beekeepers (see supporting information). Capped brood size is  
113 crucial to colony development and is relatively easy to measure in the field (Requier et al., 2017). All  
114 colonies were equipped with pollen traps that were activated for 4 consecutive days per week; pollen  
115 samples were pooled on a bi-weekly basis. Pesticide residues in pollen samples were determined using  
116 a multi-residue analysis with LC-MS and GC-MS methods (Primoris, Belgium). The method has the  
117 ability to detect and quantify > 500 pesticides and their metabolites with a limit of detection ranging  
118 from 0.003 – 0.05 mg.kg<sup>-1</sup>. LOD values for each pesticide that was found in pollen can be found in  
119 Table S1.

120 To select potentially hazardous pesticide mixtures, we followed a step-by-step procedure. We first  
121 identified the most prevalent compounds (present in at least 10 samples out of the 191). Then, we  
122 determined their potential toxicity to colonies by classifying these compounds according to their mode  
123 of action (MOA) by using the Fungicide Resistance Action Committee (2015), Insecticide Resistance  
124 Action Committee (2015) and Herbicide Resistance Action Committee (2010) MOA classifications.  
125 For each pollen sample, indexes of toxicity were assessed for each of the thirteen MOAs. To  
126 determine the indexes of toxicity, the concentrations of the 21 most prevalent compounds, were  
127 weighted by their LD<sub>50s</sub> (Johnson et al., 2013; Devillers, 2002; Stevenson, 1978; Tomlin, 2009) (Table  
128 S2). Then, the weighted concentrations of all compounds sharing the same MOA in the sample were  
129 added. Thirteen MOA indexes were used to explain the variations in capped brood growth rates via a

130 generalized linear mixed model (GLMM) that considered each MOA index as a fixed effect and the  
131 apiary and colony as random factors to account for the spatial non-independency of repeated  
132 measurements (see supporting information). The variance was allowed to differ among the different  
133 apiaries. The model was fitted using the *lmer* function of the *lme4* R-package (Bates et al., 2014).  
134 Finally, we performed a co-occurrence analysis between the compounds that grouped into the MOAs  
135 that were negatively associated with brood growth dynamics and the remaining compounds using a  
136 probabilistic model for pair-wise patterns and the *co-occur* package in R (Fig. 1) (Veech, 2013). We  
137 looked for mixtures of co-occurring compounds with prevalence above 60 % amongst the colonies of  
138 a given apiary.

139

## 140 **2.2. Pollen contamination with pesticide mixtures**

141 Blends of fresh pollen with a predominance of *Castanea* sp. were obtained from Pollenergie ®  
142 (France). The presence of pesticide residues in pollen blends was assessed by a professional food  
143 analyst via LC-MS and GC-MS methods (Primoris, Belgium). The accredited method for the analysis  
144 of 500 pesticides and metabolites in pollen has a reporting limit of 0.01 mg/Kg. No pesticide or  
145 respective metabolite was detected in the pollen blend that was then used for all experiments.

146 Pollen was contaminated with pesticide mixture solutions using a Potter Spray Tower. To contaminate  
147 pollen, 24 grams of finely ground pollen were placed on a rotating plastic disk (diameter 15.9 cm) and  
148 20 ml of the pesticide mixture solution was sprayed for 30 s depositing around 500 mg of the solution  
149 on the disk. Seeing that in preliminary trials of the contamination procedure, the concentration of  
150 pesticides recovered was generally lower than the targeted concentration (likely due to solute loss  
151 during the spraying method), to obtain an environmentally relevant concentration for each pesticide of  
152 each mixture, the mean concentration of the pesticide found in field samples was multiplied by three.  
153 These values approached the upper range of the distribution of concentrations recovered in the field  
154 survey; many close to the 90e percentile (see Tables S1 and S3 for details). Pure pesticide standards  
155 were purchased from Techlab (Saint-Julien-les-Metz, France) and used to prepare the pesticide stock  
156 solutions. Individual stock pesticide solutions were prepared using a 1:1 acetonitrile-water solution at



157 the concentrations detailed in Table S3. Pesticide mixtures were prepared by mixing stock solutions  
158 with water to obtain a final acetonitrile water ratio of 1:3. Control pollen was prepared by spraying a  
159 solution of acetonitrile-water in a 1:3 ratio.

160 Confirmation of the adequate contamination of pollen was carried out by multi-residue analysis as  
161 described above (Primoris). The amount of pesticides recovered in the pollen showed some variation,  
162 on average, of the amount added, 76.7 % was successfully recovered (Table S3), with values above or  
163 below the targeted concentration (Table S1). Nonetheless, the contamination method using the Potter  
164 Spray Tower was effective, and we were able to correctly replicate the composition and concentration  
165 of the 5 pesticide mixtures (Table S3).

### 166 **2.3. Bee exposure to pesticide mixtures**

167 Experiments were performed at the Institut National de la Recherche Agronomique (INRA) in  
168 Avignon (France) with honey bees (*Apis mellifera* L.) from local apiaries. To obtain newly emerged  
169 bees, brood frames from 3-5 colonies containing late-stage pupae were placed overnight in an  
170 incubator at 34 °C and 50-70 % humidity. The next day, newly emerged bees were pooled and groups  
171 of 75 bees were then introduced into a frame-cage compartment for a chronic exposure of 6 days to  
172 contaminated pollen (mixtures A-E) or pollen sprayed only with solvent (control treatment)(Fig. S1).  
173 Frame-cages were constructed using a wooden frame from the receiving colony and wire mesh. The  
174 41.3 × 17.8 × 2.1 cm frame had wooden separators creating six 5.4 × 13.5 × 2.1 compartments. One of  
175 the sides of the cage was closed with wire mesh and allowed confined bees to have contact with host  
176 bees through antennal contacts and trophallaxis. The other side of the cage was closed with an inner  
177 comb layer and an outer wire mesh layer. In preliminary trials, we found that allowing bees to have  
178 contact with the comb of the host colony increased the chances of acceptance after their release. Bees  
179 in each frame-cage were supplied with two water dispensers and a feeder containing control pollen or  
180 one of the treated pollen blends that were replenished every two days. In order to estimate the  
181 exposure to pesticide mixtures, pollen consumption was quantified (Fig. S2).

### 182 **2.4. Influence of pesticide mixtures on flight activity**

183 Before their introduction into frame-cages, newly emerged bees were marked with a data-matrix  
184 barcode (3 mm diameter, see graphical abstract) printed on laminated paper and glued on the thorax  
185 (Sader®). After six days of confinement, corresponding to the major period of pollen consumption, the  
186 cages were opened and barcoded bees were allowed to move freely inside and outside the hive. The  
187 activity of barcoded bees was recorded using optical bee counters at the entrance of the hive (Alaux et  
188 al., 2014). The optical bee counter consists of a camera that monitors the hive entrance and image  
189 analysis software that detects and registers the barcode. The experiment was repeated 5 times (1 in  
190 April, 3 in July and 1 in September 2016) using four different host colonies. Of the 2,250 individually  
191 marked bees, longevity and behavioral data were successfully recorded for 1,450 bees (at least one exit  
192 and entrance sequence). We attributed this loss to the loss of barcode tags prior to leaving the hive,  
193 rejection from the host colony by nest mates or death during the bee's first flight. The last detection  
194 for each barcoded bee was used to calculate bee survival. Survival curves for each treatment were  
195 fitted using the *surv* function and difference in survival amongst treatments was determined by a log-  
196 rank test using the *survdiff* function of the *survival* package in R (Therneau and Grambsch, 2013). In  
197 order to identify which contaminated pollen treatments affected bee survival, pairwise comparisons  
198 were performed with log-rank tests and the *P* values were corrected using the Bonferroni method.  
199 For the analysis of flight activity, exit-entrance sequences shorter than 1 min or longer than 240 min  
200 were excluded (not considered as foraging flights). Variations in the age at which bees accumulated 30  
201 min outside the hive, attributed to the onset of foraging (Perry et al., 2015), were analyzed with a  
202 negative binomial regression using the *glm.nb* function from the *MASS* R-package. Variations in  
203 different parameters of flight activity (number of flights, time spent outside, and hour of first flight) in  
204 response to age and treatment were analyzed using GLMM fitted by maximum likelihood using the  
205 *lme4* and *nlme* R-packages. Variations in the number of flights performed per day were fitted with a  
206 Poisson error distribution and a log link function using the *glmer* function. The time spent outside per  
207 day and the hour of the day of the first flight were fitted with a Gaussian error distribution using the  
208 *lme* function. The host colony was considered as a random factor; this random term accounted for  
209 variability due to season as different bee colonies were used in April, July and September.

## 2.5. Influence of pesticide mixtures on energetic metabolism

Because insect flight is one of the most intense and energy-demanding physiological animal processes (Dudley, 2002), the alteration of flight activity by pesticide mixtures C and D (see result section) pointed towards a perturbation of the bee's energetic metabolism. We therefore assessed the energetic metabolism of bees after six days of chronic exposure to pesticide mixtures C and D. The experiment was performed on 3 colonies in April 2017. Bees were exposed to pesticide mixtures as mentioned above, but instead of being released, they were sampled after six days of exposure, flash-frozen in liquid nitrogen and stored at -80°C. We first assessed the expression level of mitochondrion-related genes (*cox17*, *mrpl-15*, *mrpl-49*, *ndufb7*, *tim8*, *tim9*). Total RNA extraction of individual bee abdomens, cDNA synthesis and qPCR reactions were performed as in Bordier *et al.* (2017b) (n = 21 bees, 7 bees per treatment per colony). The cycle threshold (Ct) values of targeted genes were normalized to the geometric mean of the housekeeping genes actin and *eif-s8* using the comparative quantification method (delta Ct method). Primers for mitochondrion-related and housekeeping genes can be found in Bordier *et al.* (2017b) and Mao *et al.* (2017). Differences in gene expression were assessed by a one-way ANOVA followed by Tukey HSD post-hoc test (i.e. *posteriori* multiple pairwise comparisons).

We then determined the ATP level and activity of the glyceraldehyde-3-phosphate dehydrogenase (GADPH), which catalyzes the sixth step of glycolysis. Individual bee thoraxes and abdomens were homogenized in lysis buffer (10 mM NaCl, 1 % (w/v) Triton X-100, 40 mM sodium phosphate pH 7.4, protease inhibitors: 2 µg/mL antipain, leupeptin and pepstatin A, 25 units/mL aprotinin and 0.1 mg/mL soybean trypsin inhibitor), to obtain a 10 % (w/v) extract. Samples were then centrifuged at 15,000 x g for 20 min at 4 °C. 50 µl of the supernatant were used to quantify ATP using the ATPlite assay kit (PerkinElmer, MA, USA) and following the manufacturers' protocol. Luminescence was measured using a plate reader (BioTek, Vermont, USA) and ATP concentrations were quantified by comparing luminescence values to a seven point standard curve (20 µM – 1 nM). ATP assays were performed in triplicate and 16 samples per treatment were analyzed. GADPH activity was measured in eight bee thoraxes per treatment using the GAPDH Assay Kit (Sigma Aldrich). Reactions were carried out in duplicate assays and compared to a six point NADH standard curve as specified by the manufacturer.

238 Differences in ATP levels and GAPDH activity were assessed by one-way ANOVAs followed by  
239 Tukey HSD post-hoc tests.

## 240 **2.6. Influence of pesticide mixtures on foraging efficiency**

241 Newly emerged bees were paint marked on the thorax (Posca®, Japan) and introduced into frame-  
242 cages supplied with one of the treated pollen blends, as described above (control, pesticide mixtures C  
243 or D). The experiment was repeated 7 times using 5 different colonies during May and June 2017.

244 Once the paint-marked bees were 12 days old, the hive entrance was closed for 10 min and all  
245 returning paint-marked foragers were sampled. Sampling was repeated four times a day per colony and  
246 carried out during 30 consecutive days for each cohort. A total of 501 foragers were sampled. Foragers  
247 were anesthetized with CO<sub>2</sub> and their pollen loads were removed with tweezers and weighed (scale  
248 precision 0.1 mg). Their abdomens were gently pressed with soft entomological tweezers (BioQuip,  
249 USA) and the liquid oozing from the crop was collected with a microcapillary tube (20 µl) and  
250 quantified. The concentration of sugar regurgitated from the crop was measured, as a percentage of  
251 saccharose equivalent, using manual refractometers (Bellingham & Stanley Ltd, Tunbridge Wells,  
252 UK: 0–50 and 45–80 °Brix). Bees returning with liquid with 10% or less sugar content were  
253 considered as water foragers (Bordier et al., 2017a). Due to the stressful handling and potential bias in  
254 later foraging activity, sampled bees were sacrificed.

255 Variations in the number of marked foragers captured per colony and sampling period were analyzed  
256 via negative binomial regression using the *glm.nb* function from the *MASS* R-package. Variation in the  
257 type of forager (nectar, pollen, both nectar and pollen, water or no resources) was analyzed with Chi-  
258 squared tests. Differences in the amount of nectar, the concentration of nectar and the amount of  
259 pollen were analyzed with Kruskal-Wallis tests.

## 260 **2.7. Simulation of honey bee colony dynamics**

261 To assess whether the occurrence of longer-lived but less efficient bees can have detrimental effects to  
262 the colony dynamic and survival, we incorporated the effects induced by mixture C (i.e. bee survival,  
263 flight activity and foraging efficiency) into a model of bee colony dynamic, the BEEHAVE model  
264 (Becher et al., 2014). A total of 700 simulations were computed. We first calibrated the model with

265 Becher *et al.*'s (2014) initial colony settings associated with a random parameterization of four  
266 parameters (egg-laying capacity, initial population size, initial honey stock, and *Varroa* infestation) for  
267 improving model stochasticity (see supporting information). Then, we incorporated the observed  
268 pesticide mixture effects into the model through modulations of the following three parameters: the  
269 age at first foraging flight (AFF), the pollen load, and the flight velocity. Flight velocity was varied to  
270 effectively decrease the number of flights per day. We included those impacts in our simulations for  
271 different time intervals during the foraging season, i.e. from day 95 (April 5<sup>th</sup>) to day 215 (August 3<sup>rd</sup>)  
272 by increment of 30 or 60 days. During the 100 computed simulations per scenario  $\times$  interval (Table  
273 S4), we gradually *i*) increased the AFF value from the default value of 21 days (ranging from 7 to 50  
274 days) to 22.71 days (ranging from 8.71 to 52.71 days), *ii*) decreased the pollen load from the default  
275 value of 0.015 g to 0.008182 g, representing a 46 % reduction in pollen load size according to our  
276 foraging results, and *iii*) reduced the daily number of flights by decreasing the flight velocity from the  
277 default value of 6.5 m.s<sup>-1</sup> to 5.076 m.s<sup>-1</sup>, representing a reduction coefficient of  $\times 0.78$  according to the  
278 full mixture-related effect in our results. Thus, each simulation involved a level of pesticide impact  
279 according to our experimental observations, linearly increasing from no-effect (0 % of the effect) to  
280 full impact (100 % of the effect). Simulation endpoints that were deemed insufficient for colony  
281 survival, *i.e.* the risk of colony collapse, were estimated by the two following thresholds established by  
282 Becher *et al.* (2014): *i*) simulations that drop below a population size of 4,000 adult bees during the  
283 winter, and *ii*) simulations that reach a null amount of honey stock during the winter season. We  
284 analyzed the risk of colony collapse related to altered bee activity over one- and half-annual cycles  
285 using GLM with Binomial error distribution. Then, we analyzed the effects on colony dynamics using  
286 linear models by computing the change in size ( $\Delta$ ) of the adult population, the larval population, and  
287 the supplies of honey and pollen between the first and last days of altered bee activity.

288

## 289 3. Results

### 290 3.1. Mixtures of contaminants in bee pollen

291 The periodic monitoring of pesticide residues revealed that pollen samples ( $n = 191$ ) from 25 honey  
292 bee colonies distributed in four apiaries in three French regions (Midi-Pyrénées, Rhône-Alpes and  
293 Provence-Alpes Cote d'Azur) were contaminated by 71 compounds commercially used as  
294 agrochemicals (Table S1). Half of these contaminants (48 %) are used as fungicides, while 30 % as  
295 insecticides/acaricides and 13 % as herbicides. We found that four MOAs (inhibitors of the  
296 biosynthesis of methionine, inhibitors of acetylcholinesterase, compounds that interfere with signal  
297 transduction pathway, and compounds that affect cellular respiration), which represent the activity of 6  
298 compounds (chlorpyrifos ethyl, dimethoate, cyprodinil, fludioxonil, iprodione and dodine), were  
299 associated with negative brood growth rates. The co-occurrence of these six compounds with the rest  
300 of the molecules (Fig. 1) revealed four field-relevant pesticide mixtures consisting of 4 or 5 fungicides  
301 and insecticides that were chosen as candidates for experimentally testing their toxicity on honey bees  
302 (mixture A, C, D and E; Table 1). The co-occurrence matrix revealed an additional prevalent mixture  
303 of herbicides and fungicides from the Midi-Pyrénées pollen samples, which was also selected for  
304 testing (mixture B, Table 1).

### 305 3.2. Pesticide mixtures affect bee survival

306 The cumulated pollen consumption per bee was estimated at  $46.34 \pm 9.15$  mg (mean  $\pm$  SD, range of 26  
307 to 61 mg). Pollen consumption did not differ among treatments (one-way ANOVA,  $P = 0.539$ ).  
308 Depending of the pesticides, chronic exposure ranged from 0.4 – 74.5 ng ingested per bee with an  
309 average of  $12.34 \pm 13.8$  ng per bee (Fig. S2). For each molecule, the dose ingested by bees was  
310 several-fold lower than the reported oral  $LD_{50}$ ; 3,000 to 100,000 times lower for fungicides, 20 – 8,000  
311 times lower for insecticides and 180 – 9,000 times lower for herbicides (Table S2).  
312 Bee survival differed significantly among treatments, most notably after the age of 20 days (Log Rank  
313 Test:  $P = 0.0029$ , Fig. 2A). Bees lived longer as a result of the chronic exposure to pesticide mixtures  
314 C and D, consisting of one insecticide (I) and 3 or 4 fungicides (F) (mixture C: tau fluvalinate (I),

315 cyprodinil (F), difenoconazole (F), dodine (F), and fludioxonil (F); mixture D: chlorpyrifos ethyl (I),  
316 cyprodinil (F), fludioxonil (F), and iprodione (F)). Treatments with mixtures A, B and E did not affect  
317 bee survival.

### 318 **3.3. Pesticide mixtures affect flight activity**

319 Analysis of bee counter data revealed a general decrease in bee flight activity as a result of the chronic  
320 exposure to pesticide mixtures C and D, while the activity of bees fed with mixtures A, B and E did  
321 not differ from control bees (bees exposed to pollen sprayed with solvent only). Because the onset of  
322 foraging is usually preceded by orientation flights that previous studies have characterized by a  
323 cumulated duration of around 30 min (Capaldi et al., 2000), the age at which bees accumulated 30 min  
324 of flight time was used as a proxy for the onset of foraging (Perry et al., 2015). Control bees started  
325 foraging at the age of 10 days, as did bees fed with mixtures A, B and E (Fig. 2B). However, bees fed  
326 with mixtures C and D started foraging 2 days later ( $12.25 \pm 0.41$  and  $11.97 \pm 0.39$  days, respectively,  
327 mean  $\pm$  SE) than control bees ( $10.54 \pm 0.3$  days, negative binomial regression:  $P = 0.002$  and  $P =$   
328  $0.011$ ; Fig. 2B).

329 The daily number of flights was significantly lower for bees exposed to mixtures C and D (Fig. 3A,  
330 Table 2). This difference in daily activity was also reflected by the daily number of minutes spent  
331 outside the hive (Fig. 3B, Table 2). On a daily basis, bees belonging to group C and D spent  $20 \pm 4.16$   
332 and  $30 \pm 4.30$  min less outside the hive, respectively, and began their daily activities later in the day  
333 than control bees (Fig. 3B and C, Table 2).

334 On average, bees from all treatments (control and mixtures A to E) accumulated around 20 flights and  
335 1,000 flight min (Fig. S3). The cumulated flight activity (number and min. of flights) did not differ  
336 between treatments (Kruskall-Wallis rank sum test,  $P = 0.3765$ ). However, bees exposed to mixtures C  
337 and D accumulated 1,000 min of flights 6 and 4 days later than control bees, and performed 20 flights  
338 4 and 5 days later than control bees, respectively (Fig. S3).

### 339 **3.4. Pesticide mixtures affect energetic metabolism**

340 Compared to control bees, three of the six genes involved in oxidative phosphorylation we analyzed  
341 were under-expressed in one or both pesticide mixture treatments (mixtures C and D; Fig. 4A); *mrpl-*

342 *15* and *tim9* were under-expressed in the presence of both pesticide mixtures and *mrpl-49* expression  
343 was inhibited by treatment D only. Abdominal ATP levels were also lower for bees from treatment C  
344 but not from treatment D (ANOVA followed by a Tukey HSD post-hoc test:  $P = 0.041$  and  $P = 0.158$   
345 for mixtures C and D, respectively; Fig. 4B). In the thorax, we found higher levels of ATP in bees  
346 exposed to treatments C and D as compared to control bees (ANOVA followed by a Tukey HSD post-  
347 hoc test:  $P = 0.001$  and  $P < 0.001$  for mixtures C and D, respectively; Fig. 4C). Furthermore, the  
348 activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), that catalyzes the sixth step of  
349 glycolysis, was also significantly higher in the thorax of bees exposed to treatments C and D (ANOVA  
350 followed by a Tukey HSD post-hoc test:  $P = 0.026$  and  $P < 0.004$  for mixtures C and D, respectively;  
351 Fig. 4D). Taken together, these results show that pesticide mixtures C (tau fluvalinate, cyprodinil,  
352 difenoconazole, dodine, and fludioxonil) and D (chlorpyrifos ethyl, cyprodinil, fludioxonil, and  
353 iprodione) cause a perturbation of energetic metabolism.

### 354 **3.5. Pesticide mixtures affect foraging efficiency**

355 Our foraging data confirms that mixtures C and D reduce bee activity as fewer bee foragers were re-  
356 captured from these two treatments during the daily sampling sessions of  $4 \times 10$  minutes (Negative  
357 binomial regression:  $P = 0.022$ ;  $P = 0.019$  for C and D, respectively) (Fig. S4). Resource preference  
358 (nectar, pollen, both nectar and pollen, water, no resources) did not differ between treatments ( $\chi^2 =$   
359  $9.49$ ,  $df = 8$ ,  $P = 0.303$ , Fig. 5A). Of the 501 foragers re-captured, 368 were carrying nectar, of which  
360 133 belonged to the control group, and 118 and 117 to treatments C and D, respectively. The volume  
361 and sucrose concentration of nectar collected by individual foragers did not differ amongst treatments  
362 (Kruskal-Wallis Tests:  $\chi^2 = 3.50$ ,  $df = 2$ ,  $P = 0.173$  and  $\chi^2 = 4.23$ ,  $df = 2$ ,  $P = 0.121$  for the nectar  
363 volume and concentration, respectively) (Fig. 5B-C). Of the 127 pollen foragers sampled, 53 belonged  
364 to the control treatment, while 48 and 26 to treatments C and D, respectively. The size of pollen pellets  
365 foraged by bees previously exposed to mixture D did not differ from control bees ( $8.11 \pm 1.33$  mg and  
366  $8.03 \pm 0.91$  mg, respectively, Wilcoxon Rank Sum Test:  $P = 0.642$ ; Fig. 5D). However, bees exposed  
367 to mixture C carried significantly smaller pollen pellets ( $4.38 \pm 0.71$  mg, Wilcoxon Rank Sum Test:  $P$   
368  $= 0.002$ ; Fig. 5C), which represented a 46 % reduction in foraged pollen.



### 369       **3.6. Consequences of longer-lived but less efficient bees on the dynamic** 370               **and survival of simulated colonies**

371 During the different tested periods (30 or 60 days) of the foraging season (5<sup>th</sup> of April - 3<sup>rd</sup> of August),  
372 the occurrence of longer-lived but less efficient bees reduced the pollen reserves and the adult  
373 population size of the simulated colonies (Table S4 and Fig. 6A and 6B). These effects did not  
374 increase the probability of collapse in the following spring if they only lasted 30 days (short period  
375 scenarios, Table S4). Nonetheless, the effects on pollen storage and population size did reduce the  
376 survival probability of the colony if the stress lasted 60 days during June and July (from day 155 to  
377 day 215; generalized linear model-GLM with Binomial error distribution  $Z = -2.585$ ,  $P = 0.0097$ , Fig.  
378 6D). During this particular scenario, the probability of survival was reduced from  $91.8 \pm 14 \%$  to  $52.9$   
379  $\pm 34.4 \%$  (Fig. 6C).

## 380       **4. Discussion**

381 Amongst chemical pollutants, pesticides are one of the few chemical groups that are released  
382 voluntarily into the environment. Despite their benefits (protecting crops from pests), their residues  
383 permeate ecosystems, exposing non-target organisms. In order to guarantee long-term ecosystem  
384 functioning, pesticide-monitoring programs need to incorporate information regarding the effects of  
385 pesticide mixtures, even at very low doses. In testing the toxicity of field-relevant mixtures of  
386 pesticides to honey bees, we have documented a hereto-undescribed effect of pesticide mixture  
387 ingestion, the occurrence of longer-lived but less efficient bees, which is paradoxical to the increased  
388 mortality rates commonly observed upon pesticide exposure (Johnson, 2015). This phenomenon,  
389 associated with a perturbation of the energetic metabolism, might go unobserved under current  
390 pesticide risk assessment methods (short-term test on cage-confined bees), but is very likely to  
391 contribute to poor colony health and increase the sensitivity to other environmental stressors.

392 Pesticide mixtures that disturbed bee energetic metabolism and slowed down flight activity were  
393 predominantly composed of fungicides, and were found in 60 and 100 % of the colonies sampled  
394 within apiaries, highlighting their prevalence. All of the compounds of mixture C are used in apple and  
395 peach orchards (Baudet and Pringard, 2018), and the compounds of mixture D are found in vineyards

396 and cereal fields (Baudet and Pringard, 2018), indicating that such environments might not be suitable  
397 for beekeeping. This is in accordance with a recent study, which reported a high incidence of  
398 fungicides in the pollen stored by migratory colonies after orchard pollination (Traynor et al., 2016).  
399 Our study also agrees with previous European studies that have reported a high incidence of  
400 fungicides in bee pollen (Genersch et al. 2010; Porrini et al. 2010; Simon-Delso 2017). Since our  
401 study aimed to evaluate the effects of pesticide mixtures, and not to test the toxicity of each compound  
402 separately or all the possible combinations of compounds of the mixtures, we cannot attribute the  
403 observed effects to a particular compound or set of compounds. However, with the exception of the  
404 fungicides iprodione and dodine, all of the compounds of mixtures C and D, including both  
405 insecticides, were also tested in mixtures A and E at similar concentrations (Fig. S2) and not found to  
406 be detrimental to the bees. It is interesting to note that the proposed MOA of the fungicide dodine is by  
407 damaging lipid bilayers and hence interfering with cellular respiration, which could partly explain the  
408 perturbations to energetic metabolism we observed, however similar perturbations were observed with  
409 mixture D that did not contain dodine. In addition, the doses of iprodione and dodine ingested by the  
410 bees were at least 10,000 times lower than the LD<sub>50</sub>. We therefore hypothesize that the observed  
411 effects were due to the interaction of two or more molecules in mixtures C and D. Our results also  
412 underscore the importance in evaluating the interactions of fungicides with insecticides. Fungicides  
413 can interfere with the detoxification system of bees and thus synergize the effects of other pesticides  
414 (Chalvet-Monfray et al., 1996; Johnson et al., 2013; Pilling and Jepson, 1993; Poquet et al., 2016).  
415 Even if recent associations between fungicide prevalence and poor honey bee and bumble bee colony  
416 development have been reported (Bernauer et al., 2015; DeGrandi-Hoffman et al., 2013; McArt et al.,  
417 2017; Simon-Delso et al., 2014; Traynor et al., 2016), fungicides are not generally considered as a  
418 threat to bees (mainly due to their high LD<sub>50</sub> values). Nonetheless, fungicides can interfere with the  
419 honey bee's ability to detoxify xenobiotics (Berenbaum and Johnson, 2015). For instance, triazole  
420 fungicides can disrupt the detoxification of the natural occurring flavonol quercetin, a ubiquitous and  
421 abundant component of pollen, which has been shown to downregulate multiple mitochondrion-related  
422 nuclear genes (Mao et al., 2017). As a result, the combination of triazole fungicides and quercetin  
423 leads to a disruption of bee energy metabolism (Mao et al., 2017). Interestingly, the slower flight

424 activity we have observed was preceded by changes in bee energetic metabolism. Because in our study  
425 bees were fed with pollen (hence quercetin) contaminated by fungicide-dominated pesticide mixtures,  
426 similar mechanisms could be expected; especially for mixture C, which included a triazole fungicide  
427 (difenoconazole). However, we cannot rule out a direct effect of individual compounds in the mixtures  
428 on bee energetic metabolism (Campbell et al., 2016). The higher thoracic ATP levels recorded after  
429 pesticide exposure might then reflect compensatory changes in response to the decrease in abdominal  
430 ATP levels (Campbell et al., 2016). This would lead, in the long-term, to energy depletion and lower  
431 behavioral performances, considering that thoracic ATP is the energetic resource of flight muscle. This  
432 phenomenon of exhaustion is further supported by a recent study, which showed that energy  
433 disruption by quercetin decreases locomotion in zebrafish larvae (Zhang et al., 2017).

434 As the number of days a honey bee spends foraging in its life (foraging period) seems to be  
435 determined by the intensity of foraging, it has been proposed that the total flight performance of the  
436 individual is fixed (Neukirch, 1982). Our data agree with the notion of a fixed total flight performance  
437 in the honey bee as exposed bees managed to spend as much time flying (around 1000 min) as controls  
438 but prolonged by four (mix D) and six days (mix C). Considering this relationship between flight  
439 activity and longevity, it is not surprising that bees exposed to pesticide mixtures C and D outlived  
440 control bees as they exhibited a reduced flight activity. Furthermore, the transition to foraging, which  
441 is a central variable in bee life expectancy (Rueppell et al., 2007), was delayed in pesticide-exposed  
442 bees and likely contributed to extending their lives.

443 While the amount and concentration of the nectar brought back during each foraging trip was not  
444 affected by ingestion of the pesticide mixtures, pollen foraging was hindered as a consequence of the  
445 exposure to mixture C. The higher sensitivity of pollen foraging as opposed to nectar foraging has also  
446 been reported for bumblebees following an exposure to pesticides (Feltham et al., 2014; Gill et al.,  
447 2012) and for honey bees after an immune stress or parasitic infection (Bordier et al., 2018; Lach et  
448 al., 2015). Foraging for pollen is more energy consuming than foraging for nectar (Feuerbacher et al.,  
449 2003), suggesting that inefficient pollen foraging could result from impaired energetic metabolism.  
450 However, we cannot exclude that our foraging results are due to other mechanisms because mixture D  
451 did not modify pollen foraging despite affecting energetic metabolism.

452 Effective pollen foraging is of great importance to the colony; 130 mg of pollen are required to rear  
453 each larva (Brodshneider and Crailsheim, 2010). Therefore, our data on the reduced pollen-foraging  
454 efficiency agree with the selection of pesticide mixtures process used in this study; based on smaller  
455 brood areas. This was further supported by simulation of colony dynamics, which showed that  
456 exposure over 30 or 60 days to mixture C would translate into decreased pollen storage and adult  
457 population size. If the stress period occurred early in the season and during a short period of exposure  
458 (30 days), simulated colonies were able to withstand the decline in pollen storage and in adult  
459 population. However, a prolonged exposure during June and July was associated with a significant risk  
460 of colony collapse in the following spring, likely due to the reduced resilience of small colonies.

461 In conclusion, our results provide compelling evidence for lifetime effects of an early-life exposure to  
462 pesticide mixtures at low doses. This highlights the importance of examining the toxicity of pesticide  
463 mixtures that occur in the field. But most importantly, it shows that the combination of pesticide  
464 residue analysis and experimental manipulation is a promising approach for identifying pesticide  
465 residues that are a risk for non-target organisms. Cause-and-effect information is urgently needed to  
466 help policy makers improve the regulation of toxic residues and guarantee long-term ecosystem  
467 functions.

468

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477

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