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

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Abstract

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

Highlights

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

Keywords

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

1. Introduction

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

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

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

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

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

2. Methods

2.1. Identification of potentially hazardous pesticide mixtures from field surveys

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

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

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

2.2. Pollen contamination with pesticide mixtures

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

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

Before their introduction into frame-cages, newly emerged bees were marked with a data-matrix barcode (3 mm diameter, see graphical abstract) printed on laminated paper and glued on the thorax (Sader®). After six days of confinement, corresponding to the major period of pollen consumption, the cages were opened and barcoded bees were allowed to move freely inside and outside the hive. The activity of barcoded bees was recorded using optical bee counters at the entrance of the hive (Alaux et al., 2014). The optical bee counter consists of a camera that monitors the hive entrance and image analysis software that detects and registers the barcode. The experiment was repeated 5 times (1 in April, 3 in July and 1 in September 2016) using four different host colonies. Of the 2,250 individually marked bees, longevity and behavioral data were successfully recorded for 1,450 bees (at least one exit and entrance sequence). We attributed this loss to the loss of barcode tags prior to leaving the hive, rejection from the host colony by nest mates or death during the bee's first flight. The last detection for each barcoded bee was used to calculate bee survival. Survival curves for each treatment were fitted using the *surv* function and difference in survival amongst treatments was determined by a log-rank test using the *survdiff* function of the *survival* package in R (Therneau and Grambsch, 2013). In order to identify which contaminated pollen treatments affected bee survival, pairwise comparisons were performed with log-rank tests and the *P* values were corrected using the Bonferroni method.

For the analysis of flight activity, exit-entrance sequences shorter than 1 min or longer than 240 min were excluded (not considered as foraging flights). Variations in the age at which bees accumulated 30 min outside the hive, attributed to the onset of foraging (Perry et al., 2015), were analyzed with a negative binomial regression using the *glm.nb* function from the *MASS* R-package. Variations in different parameters of flight activity (number of flights, time spent outside, and hour of first flight) in response to age and treatment were analyzed using GLMM fitted by maximum likelihood using the *lme4* and *nlme* R-packages. Variations in the number of flights performed per day were fitted with a Poisson error distribution and a log link function using the *glmer* function. The time spent outside per day and the hour of the day of the first flight were fitted with a Gaussian error distribution using the *lme* function. The host colony was considered as a random factor; this random term accounted for 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.

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

2.6. Influence of pesticide mixtures on foraging efficiency

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

Once the paint-marked bees were 12 days old, the hive entrance was closed for 10 min and all returning paint-marked foragers were sampled. Sampling was repeated four times a day per colony and carried out during 30 consecutive days for each cohort. A total of 501 foragers were sampled. Foragers were anesthetized with CO_2 and their pollen loads were removed with tweezers and weighed (scale precision 0.1 mg). Their abdomens were gently pressed with soft entomological tweezers (BioQuip, USA) and the liquid oozing from the crop was collected with a microcapillary tube (20 µl) and quantified. The concentration of sugar regurgitated from the crop was measured, as a percentage of saccharose equivalent, using manual refractometers (Bellingham & Stanley Ltd, Tunbridge Wells, UK: 0–50 and 45–80 °Brix). Bees returning with liquid with 10% or less sugar content were considered as water foragers (Bordier et al., 2017a). Due to the stressful handling and potential bias in later foraging activity, sampled bees were sacrificed.

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

2.7. Simulation of honey bee colony dynamics

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

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

3. Results

3.1. Mixtures of contaminants in bee pollen

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

3.2. Pesticide mixtures affect bee survival

The cumulated pollen consumption per bee was estimated at 46.34 ± 9.15 mg (mean ± SD, range of 26 to 61 mg). Pollen consumption did not differ among treatments (one-way ANOVA, P = 0.539). Depending of the pesticides, chronic exposure ranged from 0.4 – 74.5 ng ingested per bee with an average of 12.34 ± 13.8 ng per bee (Fig. S2). For each molecule, the dose ingested by bees was several-fold lower than the reported oral LD_{50}; 3,000 to 100,000 times lower for fungicides, 20 – 8,000 times lower for insecticides and 180 – 9,000 times lower for herbicides (Table S2).

Bee survival differed significantly among treatments, most notably after the age of 20 days (Log Rank Test: P = 0.0029, Fig. 2A). Bees lived longer as a result of the chronic exposure to pesticide mixtures 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 ± 0.41 and 11.97 ± 0.39 days, respectively,
327 mean ± SE) than control bees (10.54 ± 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 ± 4.16
332 and 30 ± 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-*

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

3.5. Pesticide mixtures affect foraging efficiency

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

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

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

4. Discussion

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

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

and cereal fields (Baudet and Pringard, 2018), indicating that such environments might not be suitable for beekeeping. This is in accordance with a recent study, which reported a high incidence of fungicides in the pollen stored by migratory colonies after orchard pollination (Traynor et al., 2016). Our study also agrees with previous European studies that have reported a high incidence of fungicides in bee pollen (Genersch et al. 2010; Porrini et al. 2010; Simon-Delso 2017). Since our study aimed to evaluate the effects of pesticide mixtures, and not to test the toxicity of each compound separately or all the possible combinations of compounds of the mixtures, we cannot attribute the observed effects to a particular compound or set of compounds. However, with the exception of the fungicides iprodione and dodine, all of the compounds of mixtures C and D, including both insecticides, were also tested in mixtures A and E at similar concentrations (Fig. S2) and not found to be detrimental to the bees. It is interesting to note that the proposed MOA of the fungicide dodine is by damaging lipid bilayers and hence interfering with cellular respiration, which could partly explain the perturbations to energetic metabolism we observed, however similar perturbations were observed with mixture D that did not contain dodine. In addition, the doses of iprodione and dodine ingested by the bees were at least 10,000 times lower than the LD_{50}. We therefore hypothesize that the observed effects were due to the interaction of two or more molecules in mixtures C and D. Our results also underscore the importance in evaluating the interactions of fungicides with insecticides. Fungicides can interfere with the detoxification system of bees and thus synergize the effects of other pesticides (Chalvet-Monfray et al., 1996; Johnson et al., 2013; Pilling and Jepson, 1993; Poquet et al., 2016).

Even if recent associations between fungicide prevalence and poor honey bee and bumble bee colony development have been reported (Bernauer et al., 2015; DeGrandi-Hoffman et al., 2013; McArt et al., 2017; Simon-Delso et al., 2014; Traynor et al., 2016), fungicides are not generally considered as a threat to bees (mainly due to their high LD_{50} values). Nonetheless, fungicides can interfere with the honey bee's ability to detoxify xenobiotics (Berenbaum and Johnson, 2015). For instance, triazole fungicides can disrupt the detoxification of the natural occurring flavonol quercetin, a ubiquitous and abundant component of pollen, which has been shown to downregulate multiple mitochondrion-related nuclear genes (Mao et al., 2017). As a result, the combination of triazole fungicides and quercetin leads to a disruption of bee energy metabolism (Mao et al., 2017). Interestingly, the slower flight

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

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

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

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

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

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