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Fipronil pesticide as a suspect in historical mass mortalities of honey bees

(Short title: Fipronil and mass mortalities of honey bees)

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Abstract

Mass mortalities of honey bees occurred in France in the 1990s coincident with the introduction of two agricultural insecticides, imidacloprid and fipronil. Imidacloprid, a neonicotinoid, was widely blamed but the differential potency of imidacloprid and fipronil has been unclear because of uncertainty over their capacity to bioaccumulate during sustained exposure to trace dietary residues and thereby cause time-reinforced toxicity (TRT). We experimentally quantified the toxicity of fipronil and imidacloprid to honey bees and incorporated the observed mortality rates into a demographic simulation of a honey bee colony in an environmentally realistic scenario. Additionally, we evaluated two bioassays from new international guidance for agrochemical regulation, which aim to detect TRT. Finally, we used analytical chemistry (GC-MS) to test for bioaccumulation of fipronil. We found in demographic simulations that only fipronil produced mass mortality in honey bees. In the bioassays, only fipronil caused TRT. GC-MS analysis revealed that virtually all of the fipronil ingested by a honey bee in a single meal was present six days later, which suggests that bioaccumulation is the basis of TRT in sustained dietary exposures. We therefore postulate that fipronil, not imidacloprid, caused the mass mortalities of honey bees in France during the 1990s because it is lethal to honey bees in even trace doses due to its capacity to bioaccumulate and generate TRT. Our results provide the first evidence that recently proposed laboratory bioassays can discriminate harmful bioaccumulative substances and thereby address evident shortcomings in a regulatory system that had formerly approved fipronil for agricultural use.

Significance

New international regulatory guidelines aim to better protect bees from harmful exposures to agrochemicals used in crop protection. Our results provide the first evidence that the laboratory bioassays in these guidelines can discriminate harmful bioaccumulative insecticides that had formerly been approved for agricultural use. As a test case, we investigated insecticides implicated in the mass-mortalities of honey bees in France in the 1990s. We demonstrate that a hitherto overlooked insecticide with a strong capacity to bioaccumulate was very likely responsible.

\body Conspicuous mass mortalities of honey bees were observed in France between 1994 and 1998 (1). Their onset coincided with the introduction of two new-to-market systemic insecticides, imidacloprid (released in 1994) and fipronil (released in 1993), which were used widely on French sunflower (*Helianthus annuus*) crops (2). Imidacloprid is a neonicotinoid pesticide that disrupts the insect nervous system by acting on nicotinic acetylcholine receptors (nAChRs) (3) and fipronil is a phenylpyrazole insecticide that acts on γ-aminobutyric acid (GABA) receptors (4). Applied as seed dressings, these systemic insecticides are taken up by the growing plant and distributed throughout its tissues, including the flowers (5). Consequently, honey bees are exposed to low-level dietary residues when feeding on nectar and pollen from systemically treated bee-attractive crops (6). Despite being used across similar acreages (7), it was generally believed that the mass mortalities were caused by imidacloprid (1), but the case against imidacloprid is weak for two reasons. First, dietary imidacloprid at environmentally realistic levels does not appear to be able to cause mass mortality in honey bees. Neonicotinoid residues in the nectar and pollen of bee-attractive crops are typically less than 6 ppb (parts per billion) (8), but the consensus dose-response relationship from four previous laboratory studies (9-11) (SI Appendix, Section S1) indicates that lethality is infrequent in this range (c. <5% mortality) even after a 10-day dietary exposure and only dietary concentrations of in excess of one hundred times the environmentally realistic level cause substantial mortality (dietary concentration for 50% mortality, LC_{50} = 1750 $\mu g\ L^{-1}$, or *c*. 1350 ppb). Continuous experimental exposures of honey bee colonies to 5 pbb imidacloprid-laced syrup over six week periods under field conditions (12) found no mass mortality and report only minor sublethal impacts on cardinal indicators of colony performance, such as hive mass and the population size of adult bees.

Second, dietary imidacloprid does not appear to bioaccumulate in individual bees, which decreases the potential harmfulness of the low-level residues that typify its presence in the nectar and pollen of treated crops. Potentially, an insecticide that is present as trace dietary residues eventually may accumulate to a lethal level during a sustained exposure, which is

possible in bees because their adult lifespan and the blooming period of mass-flowering crops like sunflower and canola both extend over several weeks. However, honey bees clear ingested imidacloprid rapidly from their bodies (92% within 48 hours) (13) and the elimination half-life of imidacloprid and its toxic metabolites is approximately 24 h (14). In theory, the bodily concentration of an ingested toxicant reaches steady-state in approximately four elimination half-lives (15), so imidacloprid is unlikely to be bioaccumulative during exposures at an ecologically relevant timescale, which is measured in weeks. Further, it appears that imidacloprid does not accumulate locally at its target sites by binding irreversibly to receptors in the insect nervous system. Instead, rapid post-exposure recovery is observed in honey bees (13) and other insects including cockroaches (16), termites (17) and bumble bees (18, 19), which clearly indicates reversible binding. Taken together, this evidence suggests that it is unlikely that even a sustained exposure to dietary imidacloprid at environmentally realistic levels can be the cause of mass fatalities.

By contrast, fipronil appears more likely than imidacloprid to have caused the mass mortalities among honey bees observed in France because it is more potent in sustained exposures. After *ad libitum* feeding on dosed diets, the 10-day LD_{50} for fipronil is 3 ng bee^{-1} compared with 189 ng bee^{-1} for imidacloprid (9). Furthermore, the fact that the LD_{50} for exposed honey bees is lower in sustained than acute exposures (10-day LD_{50} = 3 ng bee^{-1} *vs*. 48-hour LD_{50} = 123 ng bee^{-1}; (9) strongly implicates bioaccumulation because each ingested unit of a bioaccumulative toxicant has greater opportunity to injure the subject in a prolonged exposure than each unit of a non-bioaccumulative toxicant, which spends a much shorter time in the subject's body. However, it is unclear whether fipronil and imidacloprid are differentially toxic in an environmentally realistic in-hive scenario involving mixed-age groups of honey bees taken directly from outdoor hives rather than in the newly emerged, well nourished, disease-free honey bees conventionally used in the laboratory tests to establish the various LD_{50} values for each toxicant. Further, it has been unclear whether the effects measured on individual bees in the laboratory are sufficient to cause mass mortality

97 in a colony. We therefore set out to experimentally quantify the differential toxicity of fipronil
98 and imidacloprid to bees taken from outdoor hives and we incorporated the emergent dose-
99 appropriate mortality rates into a demographic simulation model (20) to evaluate their
100 potential to cause colony-level impacts under an environmentally realistic scenario.

101 Our experiments also provided an opportunity to test whether the high toxicity of fipronil is
102 due to bioaccumulation. The mark of a bioaccumulative toxicant is that it is increasingly
103 injurious as the exposure is prolonged because its bodily levels increase, which is termed
104 'time-reinforced toxicity' (TRT) (21). One possible explanation for the historical mass-
105 mortalities of honey bees in France is that they were caused by the accumulation of trace
106 residues of a TRT-capable pesticide. We therefore investigated each pesticide's capacity to
107 generate TRT as suggested in proposed international guidelines for regulatory testing (22)
108 by evaluating Haber's 'constant product' rule. Specifically, we used the results of our
109 experiments to evaluate the exponent, b, in the following constant-product relationship:

110 $$Ct^b = k \qquad \text{Eq. 1}$$

111 where C denotes the concentration of the toxicant at its target site and t denotes the duration
112 of the exposure in the dose-duration combinations that produce a specified injury, such as
113 fatality. In theory, the exponent takes the value b =1 if the toxicant reaches steady-state and
114 b = 2 if the toxicant bioaccumulates (SI Appendix, Section S2). The evaluation of the
115 exponent in Haber's 'constant product' rule is a widely recognised approach in toxicology
116 (23) and risk assessment (24) that has been recently recommended for understanding bee-
117 pesticide interactions (21).

118 We additionally employed a second test for TRT based on recent EFSA (European Food
119 Safety Authority) draft guidance (25). When experimental exposures are conducted at a
120 range of doses and mortality is recorded, toxicokinetic inferences can be made by
121 comparing the ingested mass that precedes fatality across the different doses. A non-
122 bioaccumulative toxicant with a short in-body residence will cause the same injury per unit

ingested irrespective of dose (because each unit has approximately the same in-body residence), which means that each bee must ingest the same total mass of toxicant to cause its fatality irrespective of the duration of the exposure. In contrast, a bioaccumulative toxicant retained in the bee's body has longer to cause injury in the longer-lived bees feeding on lower doses, so these bees need to consume less of the toxicant in total to be killed. Hence, a second signature of TRT is evident when the fatal mass of ingested toxicant declines as the duration of exposure increases, which can be evaluated by testing the ingestion-*vs.*-longevity relationship for a negative slope.

In addition to investigating the toxicity of the focal historical compounds, imidacloprid and fipronil, we also examined two other pesticide compounds in widespread current use, thiamethoxam (a neonicotinoid) and cypermethrin (a pyrethroid). Whereas thiamethoxam has been used worldwide, ongoing concerns over bee health have led the European Union recently to ban the use of neonicotinoids and fipronil on bee-attractive crops (26), but until recently derogations in the United Kingdom have allowed some farmers to use neonicotinoids (including thiamethoxam) on oilseed rape (*Brassica napus*) (27). Meanwhile, other farmers are instead using non-systemic pyrethroid foliar sprays with active ingredients such as cypermethrin, which acts on the sodium channels in the post-synaptic membrane of insect nerve cells (28). Using data from experimental exposures, we estimated demographic mortality rates of all four candidate pesticides and evaluated their potential impact on a computer-simulated colony (SI Appendix, Section S3). We investigated whether fipronil's high potency in sustained exposures (9) emerged from bioaccumulation by testing for the two signatures of time-reinforced toxicity and by using gas chromatography-mass spectrometry (GC-MS) to establish its bioaccumulative potential. Additionally, we used our approach to confirm that neither imidacloprid, thiamethoxam nor cypermethrin generate TRT.

Results

Experimental exposure to dietary fipronil caused dose-dependent reductions in the longevity (days of exposure survived) of adult honey bees (Fig. 1; SI Appendix, Fig. S4.1) and residues in the environmentally realistic range produced a demographic effect that was consistently strong across both experiments (daily *per capita* mortality rate due to pesticide, 2013 exposure: $M_{pesticide}$ = 0.045, 2015 exposure: $M_{pesticide}$ = 0.099; SI Appendix, Table S3.1). When this effect was applied to the simulated colony, fipronil caused a mass mortality of adult bees. Specifically, the demographic simulation predicted the death of between 4000 and 9000 additional bees (between approximately 20% and 50% of the original population) over the first week of exposure to fipronil (Fig. 2a), which can account for '*un tapis d'abeilles mortes*' (a carpet of dead bees) in front of each colony, a symptom that characterised the affected French apiaries during the 1990s (29). In a prolonged exposure, fipronil caused the simulated colony to fail within two or three weeks (Fig. 2b). Laboratory exposures to fipronil exhibited both of the signatures of TRT (C-*vs.*-t relationship, regression analysis, 2013 exposure: b = 1.8 ± 0.14, 2015 exposure: b = 2.8 ± 0.12, Fig. 3a; ingestion-*vs.*-longevity relationship, Spearman's correlation analysis, 2013 exposure: *rho* = -0.92, P <0.001; 2015 exposure: *rho* = -0.90, P <0.01; Fig. 3c). Using GC-MS analysis of honey bee whole-body residues, we established that the highly toxic sulfone metabolite produced from a single fipronil-laced meal persisted undiminished in honey bees for at least six days (Fig. 4), which confirms and extends a recent 48-hour study (30). Consequently, fipronil sulfone appears very likely to bioaccumulate if the dietary intake were to be sustained. Taken together, these findings suggest that bioaccumulation of fipronil metabolites is the cause of the time-reinforced toxicity observed in our experiments.

Experimental exposure to dietary imidacloprid caused a hormetic response in the mean longevity of adult honey bees (i.e. low-dose stimulation coupled with high-dose inhibition; Fig. 1; SI Appendix, Fig S4.1). As expected, dietary imidacloprid produced only a slight increase in the level of mortality at an environmentally realistic dose with a correspondingly small demographic effect ($M_{pesticide}$ = 0.004; SI Appendix, Table S3.1) that was approximately

ten times smaller than the effect of fipronil. When this effect was applied to the simulated colony, imidacloprid caused only a small increment in the colony-wide mortality of adult bees (≈ 400 additional deaths over the first week of exposure; SI Appendix, Fig. S5.1a), which is not sufficiently large to be considered as a mass mortality. Additionally, exposure to imidacloprid had virtually no effect on colony growth (Fig. 2b). The mortality rate due to exposure to dietary imidacloprid measured in our present study corresponds very closely to the rate that can be estimated from a meta-analysis of previous laboratory studies in honey bees (SI Appendix, Section S1), which supports the general inference that trace levels of dietary imidacloprid do not cause fatality in adult workers. Also as expected, exposures to imidacloprid exhibited neither signature of TRT (C-*vs.*-t relationship, regression analysis, b = 0.4 ± 0.34, Fig. 3b; ingestion-*vs.*-longevity relationship: Spearman's correlation analysis, *rho* = 0.42, P >0.05; Fig. 3d), which indicates that it is unlikely to be bioaccumulative in honey bees.

Dietary thiamethoxam reduced the mean longevity of adult honey bees in exposures to residues in the environmentally realistic range (Fig. 1; SI Appendix, Fig. S4.1) with a moderate demographic effect ($M_{pesticide}$ = 0.0086; SI Appendix, Table S3.1) that was approximately five times smaller than the effect of fipronil. When this effect was applied to the simulated colony, thiamethoxam caused a moderate increase in the mortality of adult bees (c. 700 additional deaths over the first week of exposure, Fig. S5.1b), which suppressed colony growth (SI Appendix, Fig. S5.1c). Thiamethoxam exhibited neither signature of TRT (C-*vs.*-t relationship, regression analysis, b = 0.7 ± 0.13; SI Appendix, Fig. S6.1a; ingestion-*vs.*-longevity relationship: correlation analysis, Spearman's *rho* = 0.04, P >0.05; SI Appendix, Fig. S6.1c).

Dietary cypermethrin caused a hormetic response in mean longevity (Fig. 1; Fig. S4.1) and an environmentally realistic exposure caused a minute increase to the level of mortality ($M_{pesticide}$ = 0.00001; SI Appendix, Table S3.1) that had negligible impact on the simulated colony. Cypermethrin exhibited neither signature of TRT (C-*vs.*-t relationship, regression

203 analysis, b = 0.4 ± 0.13; Fig. S6.1b; ingestion-*vs.*-longevity relationship: Spearman's
204 correlation analysis, *rho* = 0.62, P = <0.001; SI Appendix, Fig. S6.1d). We speculate that the
205 positive slope of the ingestion-*vs.*-duration relationship for cypermethrin indicates that the
206 bees were detoxifying this substance more effectively at lower doses.

207 ## Discussion

208 Based on our findings, we postulate that fipronil is a credible cause of the mass mortalities of
209 honey bees that were associated with agricultural sunflower in France during the 1990s.
210 Fipronil can be lethal to honey bees in dietary exposures to the trace residues that typify
211 those in nectar and pollen from treated crops, due in part to its capacity to generate time-
212 reinforced toxicity (TRT).We estimate that the resulting increase in the demographic
213 mortality rate is capable of causing mass mortality among adult bees. Our present study has
214 examined in detail only a lethal endpoint, but fipronil may also have various sublethal
215 impacts, such as detrimental effects on foraging intensity and homing success (31), which
216 could further accelerate colony failure. Our hypothesis that fipronil is capable of causing
217 major impacts on honey bees is supported by the occurrence of occasional mass mortalities
218 of honey bees, such as the 2014 event that involved 172 hives across 23 apiaries in the
219 Canton of Bern, Switzerland (32). Despite the ongoing ban on the use of fipronil in
220 European agriculture, the accident in Bern arose from fipronil residues present as an
221 accidental contaminant in a batch of fungicide that had been used to treat nearby fruit trees,
222 which were foraged by honey bees. It is not yet possible to identify fipronil definitively as a
223 culprit in the French incidents because there is a lack of data to prove the historical levels
224 and prevalence of its residues in nectar and pollen, but the findings of our laboratory
225 experiments provide strong evidence that justifies a future programme of field
226 experimentation to further test the hypothesis.

227 ### Imidacloprid and mass mortalities of honey bees

228 Our results suggest that dietary exposure to imidacloprid in nectar and pollen is an unlikely
229 cause of mass mortality in adult honey bees because trace dietary residues at
230 environmentally realistic levels cause only low levels of mortality, even in sustained
231 exposures. This finding is consistent with the levels of mortality that have been reported by
232 previous researchers (SI Appendix, Section S1). Of course, some recent mass mortalities of
233 honey bees were instead caused by the release of insecticidal neonicotinoid dust created
234 when treated maize seeds were planted by pneumatic drilling machinery, such as the 2008
235 incident in Baden-Würtemberg in Germany (33), but dust emission cannot account for the
236 mass mortalities that coincided with the mid-summer bloom of French sunflower crops,
237 however, because agricultural sowing (including maize) occurs earlier in the year.

238 Imidacloprid's low toxicity in sustained dietary exposures to trace residues (12) appears to
239 be due in part to its rapid elimination by bees, which makes it unable to bioaccumulate and
240 thereby generate time-reinforced toxicity (TRT). Bees can eliminate ingested imidacloprid in
241 part because it binds reversibly to its target receptor, which is indicated by two lines of
242 evidence. First, *in vitro* experiments using radio-labelled ligands show that imidacloprid can
243 be displaced from the neuroreceptors of stable flies, *Stomoxys calcitrans* (34) (SI Appendix,
244 Section S7) and that this process can be rapid (dissociation half-life of approximately 10
245 minutes) in house flies (*Musca domestica*) (35) and aphids (*Myzus persicae*) (36). In
246 principle, similar reversible ligand-receptor binding therefore appears likely in bees. Second,
247 the timescale of post-exposure recovery (24-48 h) in honey bees (13) and bumble bees (18,
248 19) coincides with the timescale of metabolic elimination (elimination half-life $\approx$ 24 h) (13, 14,
249 19), which logically suggests that imidacloprid increasingly dissociates from its receptors as
250 detoxification reduces the concentration of its unbound form. Taken together, this collection
251 of evidence indicates that imidacloprid binds reversibly to its receptors in bees and, if so,
252 toxicodynamic-kinetic theory relating to toxicants with a short elimination half-life (15)
253 predicts the absence of TRT in experimental exposures to imidacloprid, just as we observed
254 in the present study. Our conclusion that imidacloprid fails to cause TRT is further supported

255 by the results of a previous laboratory exposure (9), which also produced results that
256 indicate the absence of TRT (C-*vs.*-t relationship, Haber exponent b = 1.1; SI Appendix,
257 Section S8).

258 While our results demonstrate that dietary imidacloprid is not lethal to honey bees at
259 environmentally relevant levels, we emphasise that this should not be taken to mean that it is
260 harmless to bees. We found that dietary imidacloprid produced a hormesis in longevity
261 (days of exposure survived) in honey bees, which is not unexpected; various chemical
262 stressors produce hormesis in insects (37) and imidacloprid itself causes hormetic
263 responses in stink bugs (38), aphids (39) and, notably, in the longevity of spider mites (40).
264 However, the increased longevity that we observed at low doses should be viewed as an
265 intoxication symptom, which is likely to displace affected honey bees from their normal
266 physiological equilibria (37, 39). Finally, we note that although our present study did not
267 consider the potentially detrimental impact of imidacloprid on other demographic variables
268 that are relevant to colony health, such as queen fecundity and larval performance, the lack
269 of mass mortalities in exposed colonies under field conditions (12) suggests that we have
270 not overlooked a crucial factor.

271 We add two further notes about the imidacloprid hormesis. First, a hormetic increase in
272 mean longevity in the low-dose range is not incompatible with a small increase in daily
273 mortality rate in the same range (such as we observed), because the increased life-span of
274 the surviving majority more than offsets the days lost by infrequent deaths. Second, the
275 increased longevity observed in our lowest doses confirms the presence of the active
276 substance and, in conjunction with dose-dependent mortality that conforms closely to
277 previous studies (SI Appendix, Section S9), validates our dose preparations.

278 **Inferences about cypermethrin and thiamethoxam**

279 Based on our findings, cypermethrin has the lowest potential for impact on colony
280 performance by directly causing adult mortality. In individual honey bees, detoxicative

281 enzyme systems can reduce harm from ingested pyrethroids (41) and preclude
282 bioaccumulative toxicity, or TRT, which may explain the low mortality rate due to
283 cypermethrin in our present study. In contrast, thiamethoxam appears capable of causing a
284 more substantive impact on colony performance by elevating the mortality rate among adult
285 workers. Nevertheless, thiamethoxam produced neither signature of TRT. It appears
286 probable that thiamethoxam does not bioaccumulate in honey bees because it is subject to
287 detoxicative metabolism, like the closely similar neonicotinoid clothianidin (42). Performing
288 the analytical chemistry to clarify the toxicokinetics of thiamethoxam in honey bees is a
289 target for future research. The relatively low levels of mortality in adult honey bee workers
290 caused by dietary exposures to thiamethoxam and cypermethrin do not preclude harmful
291 sublethal effects on colony performance (43).

292 **Future research and regulatory implications**

293 We used laboratory toxicology and demographic simulation to predict the potential of four
294 dietary pesticides to produce mass mortality of adult honey bees. The postulated absence
295 of mass mortality in environmentally realistic exposures to 5 ppb imidacloprid is already
296 supported by the outcome of in-hive exposures under field conditions (12), but further
297 research is necessary to provide a similar experimental evaluation of the other predictions,
298 especially the proposition that fipronil has the capacity to cause mass mortality in honey
299 bees. Various insects besides honey bees forage on the flowers of pesticide-treated crops,
300 including other kinds of bees that may be affected more strongly (44). Consequently, other
301 species should be tested to assess more broadly the impacts of pesticides on farmland
302 insect faunas.

303 When Haber's Rule successfully describes the manifestation of toxic injuries, it suggests
304 underlying proportionalities between exposure concentration, bodily concentration and the
305 accrual rate of injury. We speculate that these proportionalities arise when physical
306 processes (e.g. diffusion, concentration-dependent association-dissociation of ligand-

307 receptor complexes) fundamentally determine the levels of toxic effects. In itself, however,
308 Haber's Rule cannot elucidate the details of pharmacological mechanisms and a
309 comprehensive theory of bee-pesticide toxicology, e.g. pharmacokinetic/pharmacodynamic
310 (PK-PD) models, remains a goal for future research.

311 The potentially severe impact of dietary fipronil highlights the need to identify agrochemicals
312 that cause TRT before they are used widely in agriculture because TRT enables trace
313 contaminants to become disproportionately harmful by sustained exposure. Formerly,
314 international regulatory procedures for the risk assessment of plant protection products have
315 relied on short-term laboratory exposures of honey bees (so-called 'first tier' tests), which do
316 not take account of the possible harm that results from TRT during realistically sustained
317 exposures. Our findings illustrate the potential value of a bioassay aimed at revealing TRT.
318 Specifically, we show that TRT can be detected both by evaluating the exponent of Haber's
319 Rule (i.e. evaluation of b in the log(t)-*vs.*-log(C) relationship) and by testing for exposure-
320 dependence of the lethal dose (i.e. evaluation of the ingestion-*vs.*-longevity relationship),
321 which endorses the value of these analyses as specified in the newly formulated draft
322 guidelines issued by both the European Food Safety Authority (EFSA) for risk assessment in
323 bees (25) and the OECD (22). By explicitly including an evaluation of TRT due to dietary
324 exposure, future risk assessments will enable regulators to better protect farmland bees and
325 the valuable ecosystem services that they provide in pollinating crops and wild flowers.

326

Materials and Methods

Honey bee demographic model

To evaluate the impact of dietary pesticides on honey bee colonies, we simulated the population dynamics of a control (unexposed) colony using a published demographic model (20) and then perturbed the mortality rate according to effects that we quantified experimentally. The previous application of the model to a toxicological perturbation (45) investigated only the loss of intoxicated foragers through homing failure, but we instead explored the case where all adult bees experience an elevated rate of mortality by feeding on either nectar or stored honey that contains a dietary pesticide. We therefore modified the original model to apply mortality due to pesticide to all adult workers in the colony (Fig. S3.1). The population dynamics of the control colony were described using previously determined parameter values [L = 2000, alpha = 0.25, theta = 0.75 (20); M_B = 0.154 (46); w = 22000 (46)(47)] so that its population of bees increased by approximately 25% over 30 days from an initial size of 18000 (13500 hive bees, 4500 foragers), which simulates the rates of development typical in France coincident with the blooming of sunflower and canola (46).

The model of Khoury *et al.* was modified (SI Appendix, Fig. S3.1) so that foragers die at a rate, $M_{B+P} = M_{total}$, that compounds the baseline rate, $M_B = M_{base}$, and the rate due to pesticide exposure, $M_{pesticide}$ (see Eq. S3.1). Hive bees die only when exposed to pesticides, at a rate of $M_P = M_{pesticide}$. Values of $M_{pesticide}$ for each pesticide were determined from experimental toxicity data (SI Appendix, Section S3).

We simulated a colony's exposure to each of four dietary pesticides by perturbing the mortality rate according to our experimental observations. To estimate the *per capita* daily mortality rate of bees feeding on each diet, we used the mean proportion dying daily, which was calculated across the time span for which the total number of experimental bees alive was three or more individuals. To determine the pesticide mortality rate applied in the demographic model, we use the environmentally realistic residue concentrations of 5 ppb for

353 imidacloprid, thiamethoxam and fipronil, and 100 ppb for cypermethrin (SI Appendix, Section
354 S3.3).

355 For predicting the number of dead bees found outside a hive (Fig. 2a; SI Appendix, Fig.
356 S5.1a,b), we assume that 2.5% of natural mortalities in control colonies occur at the hive
357 (47) whereas under pesticide exposure all mortalities occur at the hive.

358 **Testing for time-reinforced toxicity**

359 Imidacloprid was obtained as a solution in acetonitrile (analytical standard, PESTANAL®,
360 Sigma Aldrich Co. LLC; product code: 46341). A vacuum concentrator (ScanSpeed MaxiVac
361 Beta; LaboGene ApS, Lynge, Denmark) was used to completely remove the acetonitrile
362 solvent and the imidacloprid was dissolved in deionised water to form a stock solution of 10
363 mg L^{-1}. Thiamethoxam, fipronil and cypermethrin (analytical standards, PESTANAL®, Sigma
364 Aldrich Co. LLC; product codes: 37924, 46451, 36128, respectively) were dissolved in water
365 (thiamethoxam) and acetone (fipronil and cypermethrin) to form stock solutions (10 mg L^{-1},
366 10 mg L^{-1} and 400 mg L^{-1}, respectively) before being combined with 50% w/v aqueous sugar
367 solution (Attraker: 1.27 kg L^{-1} fructose/glucose/saccharose solution; Koppert B.V., Berkel en
368 Rodenrijs, Netherlands). Doses of cypermethrin contained a maximum of 0.95% acetone
369 v/v, reducing with cypermethrin concentration (control doses contained 0.95% acetone v/v).
370 Doses of fipronil contained a maximum of 1.25% acetone v/v, reducing with fipronil
371 concentration (control doses contained 1.25% acetone v/v).

372 Adult worker honey bees (*Apis mellifera*) of various ages were obtained from two well-
373 managed apiaries in Devon, United Kingdom, which were separated by over 25 km. We
374 conducted exposures on fipronil, thiamethoxam, and cypermethrin using bees from apiary A,
375 which were collected in June and July 2013. We made a single collection from apiary B in
376 August 2015, which we randomly split to make a comparative series of exposures to
377 imidacloprid and fipronil. Each experiment involving a single pesticide was conducted on
378 bees taken from a single hive in an apiary, which were collected by opening the hive and

379 scooping them from the top boards and shaking them from the frames. Newly-eclosed bees
380 were not used because we wanted to determine the effects of pesticide on a
381 demographically representative sample of adults, which is an environmentally realistic
382 scenario. Honey bees were caged in groups of 10 (cage dimensions: approx. 0.10 m
383 diameter × 0.04 m height) in plastic containers, with seven replicate cages per dose. Sample
384 sizes were not chosen *a priori* based on a statistical power, but they equal or exceed those
385 used in comparable published studies. Cages of bees were randomly assigned to doses and
386 randomly positioned in the laboratory with respect to dose. Bees were kept in a semi-
387 controlled environment (daily mean temperature ± S.E.= 24.4 °C ± 0.18; mean relative
388 humidity = 35.9 % ± 0.76; 12:12 hours of low-light:darkness), but bees were capable of
389 maintaining warmer body temperatures (> 30 °C) due to non-flight thermogenesis (SI
390 Appendix, Section S10). Bees were fed *ad libitum* on syrup containing either imidacloprid
391 (dosages: 0.00, 8.00, 20.00, 50.00, 125.00, 187.50, 250.00, 500.00, 1000.00 or 2000.00 µg
392 L^{-1}), thiamethoxam (0.00, 8.00, 20.00, 50.00, 125.00, 218.75 or 312.50 µg L^{-1}), fipronil (0.00,
393 3.20, 8.00, 20.00, 50.00, 87.50 or 125.00 µg L^{-1}) or cypermethrin (0.00, 0.78, 1.95, 4.88,
394 12.21, 21.36, 30.52, 41.99, 53.46 or 64.94 mg L^{-1}). Each cage received one of the doses,
395 whose collective range spanned and exceeded the environmentally realistic concentrations.
396 Bees were monitored daily for mortality (corpses were removed daily) and syrup
397 consumption was measured daily by weighing syrup feeders for the first 10 days of
398 treatment, and every 2-3 days thereafter (SI Appendix, Section S11). Syrup feeders were
399 replaced completely at least every two or three days to ensure a continuous *ad libitum*
400 supply.

401 The power law relationship between dietary concentration of pesticide and mean longevity
402 was fitted on log-transformed axes and the slope of the relationship (parameter b) was
403 determined by linear regression. We used mean longevity because a sample mean is
404 inherently less prone to statistical error as a measure of central tendency than the median
405 (SI Appendix, S2.6). Haber's Rule (Eq. 1) predicts an infinite lifespan for exposed subjects

as dose (C) approaches zero. However, this model of toxicity cannot fit lifespan data from experiments with real animals because lifespan is constrained by the organism's senescence at the lowest doses and so the dose-longevity relationship is necessarily hockey stick-shaped (SI Appendix, Fig. S2.7). In order to objectively exclude the non-linearity, we used the lower confidence interval on the longevity (days of exposure survived) of control bees to define the range used to fit the straight-line log(C)-vs.-log(t) relationship of Haber's Rule (SI Appendix, Section 2.7). Note that we were evaluating whether the longevity of an individual dosed cage belonged to the control population, hence the confidence interval was calculated using the SD and not the SE.

Honey bee whole-body residue assay

Of the four focal pesticides, only fipronil was tested for bioaccumulation within honey bee bodies as it alone exhibited time-reinforced toxicity. Our methods were based on the OECD guideline (No. 213) for the honey bee acute oral toxicity test (48). Adult worker honey bees of varied age were starved for two hours, each cage of 10 bees was then fed 200 µL of either control syrup or syrup containing fipronil at a concentration of 145 µg L^{-1} (i.e. 2.9 ng bee^{-1}). Cages were sampled over a 6 day period (full methods and results, SI Appendix, Section S12). Residues of fipronil and its main toxic metabolite (fipronil sulfone) were measured in samples each comprising the bees collected from a single cage using gas chromatography mass spectrometry (GC-MS) (SI Appendix, Section S13). Bees fed control syrup were analysed only for residues of fipronil sulfone.

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Footnotes

The datasets generated and analysed during the current study are available in the University of Exeter ORE repository (https://ore.exeter.ac.uk/repository/) using accession number XXXXXXX.

P.J.H carried out the laboratory work and statistical analysis, co-designed the study and co-wrote the manuscript; A.J carried out the GC-MS analysis; C.R.T contributed to methods and co-wrote the manuscript; J.E.C conceived the study, co-designed the study and co-wrote the manuscript. All authors gave final approval for publication.

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Figure legends

Figure 1. Dose-dependent variation in the longevity of adult honey bees during dietary exposures to four pesticides (Fip = fipronil; Imi = imidacloprid; Tmx = thiamethoxam; Cyp = cypermethrin). Each datum indicates the relative longevity (y-axis: mean days of exposure survived relativized against undosed controls) observed in an experimental cage at a given dietary dose (x-axis: dietary concentration of toxicant in μg L^{-1}). Error bars indicate 95% confidence intervals (1.96 SEM, n = 7 cages per level of dose). Fipronil exposures were made twice (2013 = ●, 2015 = ○); the effects of low fipronil doses are strongest in relative terms in 2015 because of the greater longevity of the bees, which apparently afforded TRT a greater opportunity to develop; mean longevity of controls, 2013 = 7.3 days; 2015 = 21.2 days.

Figure 2. Impacts of dietary exposure to fipronil and imidacloprid on a simulated honey bee colony. Upper panel (a): Model predictions of the number of dead bees (y-axis: number of dead adult worker bees) to be found outside a hive over a seven day period (x-axis: time in days) under control conditions (symbol: filled circles) and during environmentally realistic dietary exposures to fipronil (squares, filled = 2013 experiment, open = 2015). Lower panel (b): Model predictions of colony size (y-axis: number of adult worker bees) over a seven week period (x-axis: time in days) under control conditions (symbol: filled circles) and during environmentally realistic dietary exposures to imidacloprid (open circles) or fipronil (squares, filled = 2013 experiment, open = 2015). The dashed line indicates the assumed minimum for colony survival. Data points of control and imidacloprid-exposed colonies have been slightly shifted in the x-plane for ease of inspection.

Figure 3. TRT indicators for fipronil and imidacloprid. Panels (a) and (b): fipronil and imidacloprid evaluated for time-reinforced toxicity by their C-vs-t relationships. Each datum indicates the mean longevity (days of exposure survived) in a cage of dosed bees. Separate experiments involving fipronil are indicated by closed (2013) and open (2015) symbols.

586 Fitted curves show log(C)-*vs*-log(t) relationships between dietary concentration (y-axis: C, µg
587 L^{-1}) and time-to-effect (x-axis: t, mean time until death of honey bees in an experimental
588 cage). Panels (c) and (d): fipronil and imidacloprid evaluated by their ingestion-*vs*-longevity
589 relationships. Each datum represents a single cage of honey bees based on: the total mass
590 of toxicant consumed by the bees before their deaths (y-axis: mass ingested, ng); and the
591 mean longevity of the exposed bees (x-axis: mean days of exposure survived). Separate
592 experiments involving fipronil are indicated by closed (2013) and open (2015) symbols.
593 Only fipronil produced significant negative trends (Spearman correlation analysis, $P < 0.001$).
594 These data include only cages of dosed bees where the reduced longevity could be
595 attributed to toxicity (see Supplemental Information Fig. S2.8).

596 **Figure 4.** Time-course of whole-body residues of fipronil (solid line) and its sulfone
597 metabolite (dashed line) in honey bees after a single fipronil-laced meal. Body residues (y-
598 axis: mean ng bee^{-1}) were measured in bees sampled separately at intervals over a six-day
599 period (x-axis: days since dose) after a single acute dietary exposure to fipronil (20 µL bee^{-1}
600 of syrup with 145 µg fipronil L^{-1}, or *c*. 3 ng bee^{-1}). Day = 0 indicates samples collected
601 immediately after dosing and Day = -1 indicates the estimated initial fipronil ingestion. Error
602 bars denote ± 1 SEM. Mean residues are connected for ease of inspection only.
603 Concentrations in undosed bees were less than 0.02 ng bee^{-1} fipronil and 0.11 ng bee^{-1}
604 fipronil sulfone.

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