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Supporting Information for

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

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Supplementary Information Text

Section S1: Meta-analysis of previously published data on total mortality in 10-day exposures of honey bees to dietary imidacloprid

Dietary imidacloprid at environmentally realistic levels does not appear to be able to cause mortality in honey bees. Neonicotinoid residues in the nectar and pollen of bee-attractive crops are typically less than 6 ppb (parts per billion), but the consensus dose-response relationship from four previous laboratory studies (Fig. S1.1) 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 mortality (dietary concentration for 50% mortality, LC_{50} = 1750 μg L^{-1}, or c. 1350 ppb).

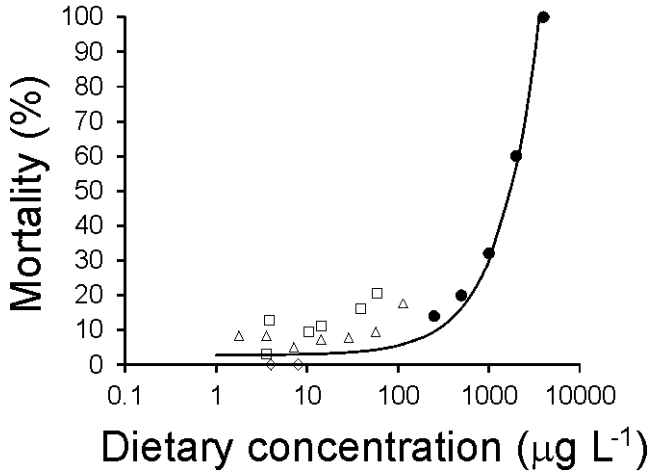

Fig. S1.1. ***Dose-dependent mortality during sustained (10 day) exposure to dietary imidacloprid in previously published studies.*** *Y*-axis: proportion of honey bees dead after 10-day exposure in relation to concentration of imidacloprid in feeder syrup (*x*-axis: μg L^{-1}). Symbols: ● Defra (2007); Decourtye et al. (2003): winter bees = □, summer bees = △; ◇ Moncharmont *et al.* (2003). Data are adjusted for control mortality by Abbott's correction.

We can use the consensus dose-mortality relationship (Fig. S1.1) to check our experimentally estimated *per capita* daily mortality rates as follows. Pesticide-dependent mortality across an exposure of *d* successive days (*M*%) is given by:

$$M\% = 100 * \sum_{i=1}^{d} \left(1 - M_{pesticide}\right)^{d} * M_{pesticide} \qquad \text{Eq. S1.1}$$

The best-fit consensus dose-response relationship (Fig. S1.1) indicates that the overall 10-day mortality in worker honey bees due to dietary imidacloprid toxicity 5 ppb is 2.9%. Using Eq. S1.1, the daily *per capita* mortality rate to produce 2.9% mortality in a 10-day exposure is $M_{pesticide} = 0.003$, which is closely similar to our experimentally determined mortality rate ($M_{pesticide} = 0.0042$), which would itself produce 4.1% mortality.

References

Decourtye, A, Lacassie, E & Pham-Delègue, MH (2003) Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season. *Pest Manag. Sci* 59:269-278.

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Section S2: Evaluation of the Haber exponent (*b* =1 vs. *b* = 2) as an indicator of bioaccumulation

Theoretical basis

If the pharmacokinetics of the toxicant in an animal's body are governed by a simple compartment model (see below), then dose-duration combinations in time-to-effect experiments will conform to a 'constant product' rule known as 'Haber's Rule':

$$Ct^b = k \qquad \text{Eq. S2.1}$$

Toxicants that are not bioaccumulative (i.e. whose internal concentration rapidly achieves steady state) will conform to Eq. S2.1 when the exponent takes the value *b* =1, and perfectly bioaccumulative toxicants will have *b* =2.

Hence, a suitable test for time-reinforced toxicity involves fitting Eq. S2.1 to a time-to-effect dataset and determining the value of the Haber's exponent, *b*. The basis and operation of this test is described below.

S2.1 Single compartment pharmacokinetics

Assume that the pharmacokinetics of the toxicant in an animal's body are governed by a simple compartment model (Fig. S2.1). The animal ingests the toxicant at a dose rate of *d* ng d^{-1} and assume that the animal's detoxification enzyme system has surplus capacity, which means that the rate of the detoxification is proportional to the internal concentration of the toxicant, ϕ.

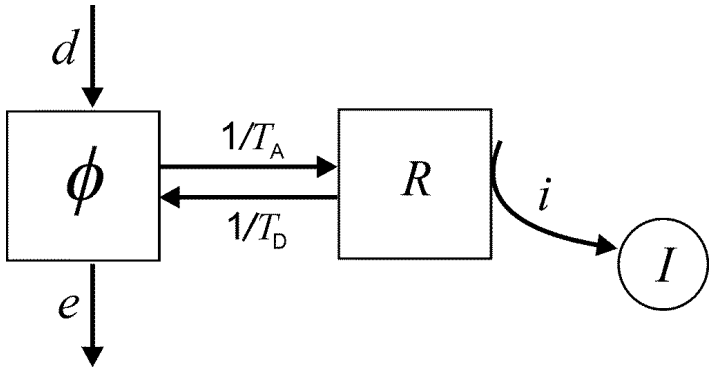

Figure S2.1. ***Compartment model of pharmacokinetics during dietary exposure to a toxicant***

Hence, the toxicant is detoxified metabolically (or otherwise eliminated from the animal's body) with first order dynamics at a rate of $e\phi$ ng d^{-1}. Let R denote the concentration of target receptors bound by the toxicant and assume that the formation of the toxicant-receptor complex is governed by coefficients of association and dissociation, denoted T_A and T_D respectively so that the rate at which the toxicant binds to receptors is R/T_A., etc. Assume that the animal incurs irreversible injury at a daily rate Ri. The total injury incurred by the organism is denoted by circular box I (the circle is used to distinguish a box that accumulates an effect from one that accumulates a mass) and the oblique arrow into the circular box indicates transfer of influence, not mass.

S2.2 Haber's exponent in a non-bioaccumulative toxicant

When a toxicant binds reversibly to its target site and is susceptible to catabolic breakdown and elimination, then during a sustained dietary exposure the continuous and opposing actions of ingestion and elimination will establish a 'steady state' concentration inside the organism and so the internal concentration, ϕ, is constant. Since R is proportional to ϕ, R is also constant over time and injury accrues at a constant rate.

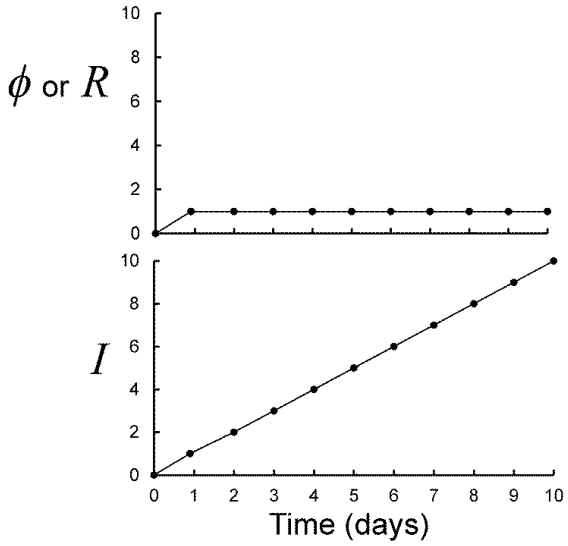

Figure S2.2. ***Pharmacokinetics and injury in a non-bioaccumulative toxicant. In this hypothetical discrete-time example: d = e = 1; $T_A = T_D = 1$.***

Hence, $I \propto t$ and $kI = \phi t$, where t denotes the duration of the exposure. If the daily rate of injury resulting from this steady state is constant, the simple pharmacokinetic compartment model of toxic load predicts that the accumulated total injury, I, is proportional to the duration of the exposure (Fig. S2.2).

The total injury across the exposure, or toxic load, is proportional to the area under the curve (AUC) of the plot of ϕ over time, which can be visualized as a rectangular geometry with area $\phi \times t$ (Fig. S2.3).

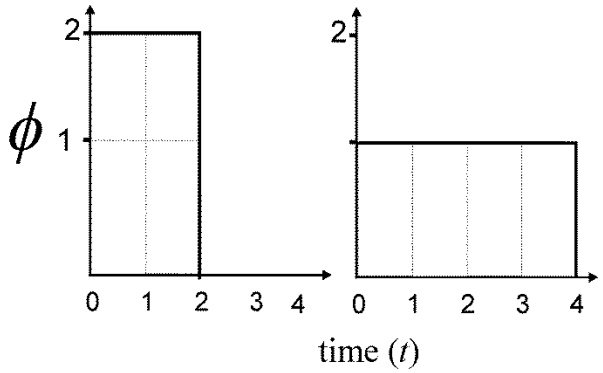

Figure S2.3. ***Toxic load in a non-bioaccumulative toxicant and Haber's Rule for a hypothetical example of two groups of animals that feed separately for four days on diets whose toxicant concentrations differ by a factor of α = 2.***

Consider two groups of animals that feed separately on diets whose toxicant concentrations differ by a factor of α (i.e. $C_1 = C_2 / \alpha$); in this hypothetical example (Fig. S2.3), α = 2. Assume that the feeding rates on the diets are equal and so the equilibrium internal concentration of toxicant will be proportional to the dietary concentration:

$$\phi \propto C \qquad \text{Eq. S2.2}$$

If the animals on the more toxic diet have an internal concentration of toxicant of ϕ_1, those that feed on the less toxic have $\phi_2 = \phi_1/\alpha$. Assume that animals feeding on the more toxic diet reach a given level of injury (toxic load) in t_1 days and those in the less toxic diet reach the same level in t_2 days (in this hypothetical example, $t_1 = 2$ days). Since the AUCs have rectangular geometry, then for both groups to experience the same injury, those on the less toxic diet must be exposed for $t_2 = \alpha t_1$ days (i.e. $t_2 = 4$ days). Formally, we can write:

$$\emptyset_1 t_1 = \frac{\emptyset_1}{\alpha} \times t_2 = \frac{\emptyset_1}{\alpha} \times \alpha t_1 \quad \text{Eq. S2.3}$$

The proportionality relationships of Eq. S2.3 imply:

$$C_1 t_1 = \frac{C_1}{\alpha} \times t_2 = \frac{C_1}{\alpha} \times \alpha t_1 \quad \text{Eq. S2.4}$$

Simplification of Eq. S2.4 and generalisation for all conforming *C* and *t* combinations yields $Ct = k$. Hence, subjects exposed to perfectly non-bioaccumulative toxicants in appropriate 'time-to-effect' experiments will exhibit outcomes that conform to a constant-product rule of $C^b t = k$ where $b = 1$. Taking logarithms of both sides of $Ct = k$ and rearranging yields:

$$\log(C) = -1 \log(T) + \log(k) \quad \text{Eq. S2.5}$$

Therefore, a non-bioaccumulative toxicant delivered in a time-to-effect experiment will produce a *C-vs.-t* relationship with a slope of -1 on log-log axes. Hence, in the constant-product relationship, $Ct^b = k$, the exponent takes the value $b=1$, which reflects a simple proportionality relationship.

This proportionality under steady-state conditions means that toxicological experiments on such a system will find that halving the dose doubles the duration of the exposure that is required to achieve a given level of injury or effect.

S2.3 Haber's exponent in a bioaccumulative toxicant

When the toxicant is not susceptible to catabolic breakdown and elimination, then during a sustained dietary exposure continuous ingestion will cause an accumulation of toxicant inside the organism and ϕ increases over time. Since *R* is proportional to ϕ, *R* also increases over time and injury accrues at an increasing rate as exposure progresses. Specifically, the organism's internal concentration at the target site rises during the exposure as the toxicant bioaccumulates, the rate of injury increases with time and so the accumulated total injury is not proportional to exposure time, but instead increases quasi-exponentially as a power function (Fig. S2.4), which is 'time reinforcement'.

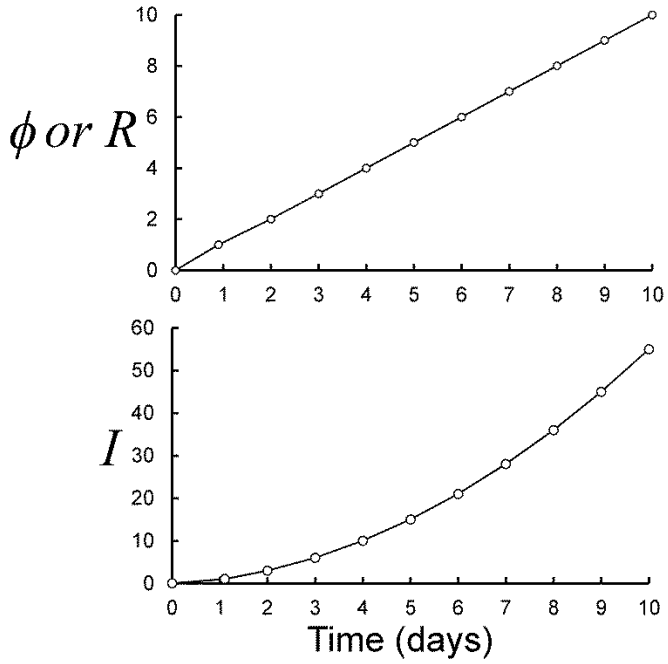

Figure S2.4. ***Pharmacokinetics and injury in a bioaccumulative toxicant. In this hypothetical discrete-time example: d = 1; e = 0; $T_A = T_D = 1$.***

Given constant ingestion of a bioaccumulative toxicant, let the internal concentration at time *t* be given by:

$$\phi = C\beta t \qquad \text{Eq. S2.6}$$

Here, the daily rate of ingestion of the toxicant, *d* ng d^{-1}, is obtained as the product of the dietary concentration, *C* ng g^{-1}, and the daily feeding rate, β g diet d^{-1}. The total injury across the exposure, or toxic load, is proportional to the area under the curve (AUC) of the plot of ϕ over time (Fig. S2.5), which can be visualized as a triangular geometry with area $0.5t \times \phi$ (i.e. half base × height).

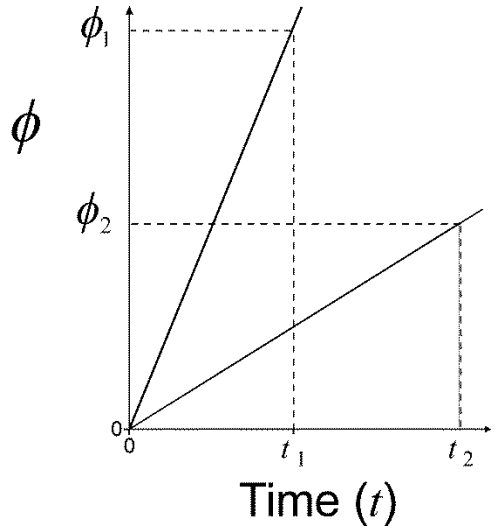

Figure S2.5. ***Toxic load in a bioaccumulative toxicant.***

As before, consider two groups of animals that feed separately on diets whose toxicant concentrations differ by a factor of α. If the feeding rates (β) on the diets are equal, the animals on the more toxic diet have an internal concentration of toxicant = $C_1\beta t_1$ and those on the less toxic diet have $C_2 = (C_1/\alpha)\ \beta t_2$. Since the AUCs have triangular geometry, then for both groups to experience the same injury we require:

$$0.5t_1 \times C_1\beta t_1 = 0.5t_2 \times \frac{C_1}{\alpha}\beta t_2 \qquad \text{Eq. S2.7}$$

Simplification yields:

$$t_1^2 = \frac{t_2^2}{\alpha} \qquad \text{Eq. S2.8}$$

Multiplying both side by C_1 yields:

$$C_1 t_1^2 = \frac{C_1}{\alpha} t_2^2 \qquad \text{Eq. S2.9}$$

Recall that the internal concentrations differ by a factor of α, so that we can write:

$$C_1 t_1^2 = C_2 t_2^2 \qquad \text{Eq. S2.10}$$

Generalisation for all conforming C and t combinations yields $Ct^2 = k$. Hence, subjects exposed to perfectly bioaccumulative toxicants in appropriate 'time-to-effect' experiments will exhibit outcomes that conform to a constant-product rule of $Ct^b = k$ where b =2.

Taking logarithms of both sides of $Ct^2 = k$ and rearranging yields:

$$\log(C) = -2\log(T) + \log(k) \qquad \text{Eq. S2.11}$$

Therefore, a bioaccumulative toxicant delivered in a time-to-effect experiment will produce a *C-vs.-t* relationship with a slope of -2 on log-log axes.
Hence, in the constant-product relationship, $Ct^b = k$, for a bioaccumulative toxicant the exponent takes the value b =2, which reflects time reinforcement.

S2.4 Graphical evaluation of the Haber exponent

It is straightforward to test for time-reinforced toxicity (TRT) by evaluating *b* using data from a series of 'time-to-effect' experiments that quantify the exposure durations required to produce a specified level of injury in experimental subjects under various doses. After conducting exposures at various doses, a suitable test for TRT involves fitting the *C-vs.-t* linear relationship (Xiao *et al.* 2011) and determining its slope on logarithmic axes (Bliss 1941), which estimates parameter *b* (Eq. S2.1) because:

$$\log(C) = -b[\log(t)] + \log(k) \qquad \text{Eq 2.12}$$

S2.5 Choice of x-y orientation and a comment on error structure

With the same data, it is possible to evaluate either $C^a t = k$ or $Ct^b = k$ (Miller, Schlosser & Janszen 2000). We have chosen to evaluate the exponent on *t* because it emphasises 'time reinforcement'. However, it is straightforward to show that $a = 1/b$, so our results are readily compared with studies that evaluate exponent *a*.
Dose-dependence is assumed *a priori*, so there is no requirement to test the *C-vs.-t* relationship for significance by regression. Consequently, whether *C* or *t* are the *x*-axis is statistically immaterial because there are no concerns about requiring the error structure to approximate a parametric statistical distribution. For the specific practical purpose of evaluating the Haber exponent, least-squares curve fitting in either orientation will produce $a \approx 1/b$, as required.

S2.6 Index of time-to-effect (days of exposure survived): median vs. mean

The median time-to-lethality (or days of exposure survived), LT_{50}, is used conventionally as the proxy for *t* in log(C)-vs.-log(t) analysis. However, it is a statistical fact that estimators of central tendency based on medians are less precise than those based on means (Wonnacott & Wonnacott 1972). Monte Carlo computer simulation of our

experiment unit (a cage of 10 honey bees) demonstrates that LT_{50} becomes increasingly imprecise as an estimator as longevity increases (Fig. S2.6) because when individual fatalities occur stochastically over an increasingly widely-spaced interval, the time of the fifth bee's death in a cage of 10 individuals becomes highly variable. In our experiments, therefore, sampling variation in LT_{50} was particularly influential during long-lasting exposures (e.g. low doses of imidacloprid and cypermethrin). We therefore used the mean longevity (days of exposure survived) instead of the median in our analyses, which was possible because we observed each cage until all individuals died.

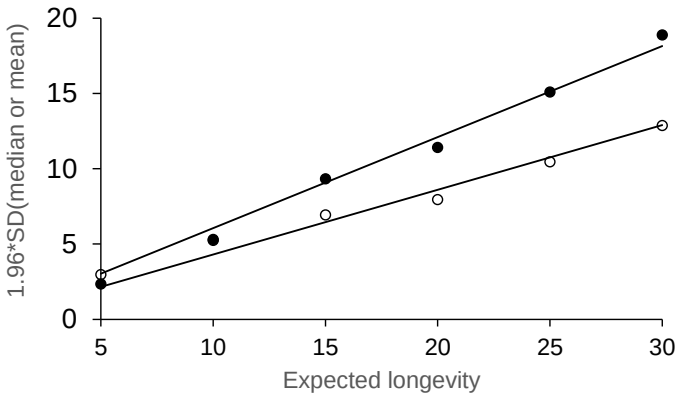

Figure S2.6. ***Sampling variation in median longevity*****.** The likely range (95% CI) of honey bee longevity in an experimental cage is greater when time-to-effect is measured by the median longevity (LT_{50}, ●) compared to the mean longevity (○). The differential increases as bees live longer (*y*-axis: expected longevity). Monte Carlo simulation is of cages of 10 bees where each individual dies on the i^{th} day with probability *p*; expected longevity = 1/ *p*.

S2.7 The influence of senescence on the C-vs.-t relationship (*the 'hockey stick' problem***)** Mathematically, Haber's Rule (Eq. S2.1) predicts an infinite lifespan for bees (*t*) as dose (*C*) approaches zero because the *Ct* product must keep the right-hand side of Eq. 2.1 constant:

$$Ct^b = k \qquad \text{Eq. S2.1}$$

In experiments with real animals, however, this model of toxicity (Eq. S2.1) will not fit data that are constrained by the organism's lifespan, which causes death by senescence at the lowest, sub-toxic doses. In reality, therefore, the observed *C-vs.-t* relationship can be hockey stick-shaped (Fig. S2.7a).

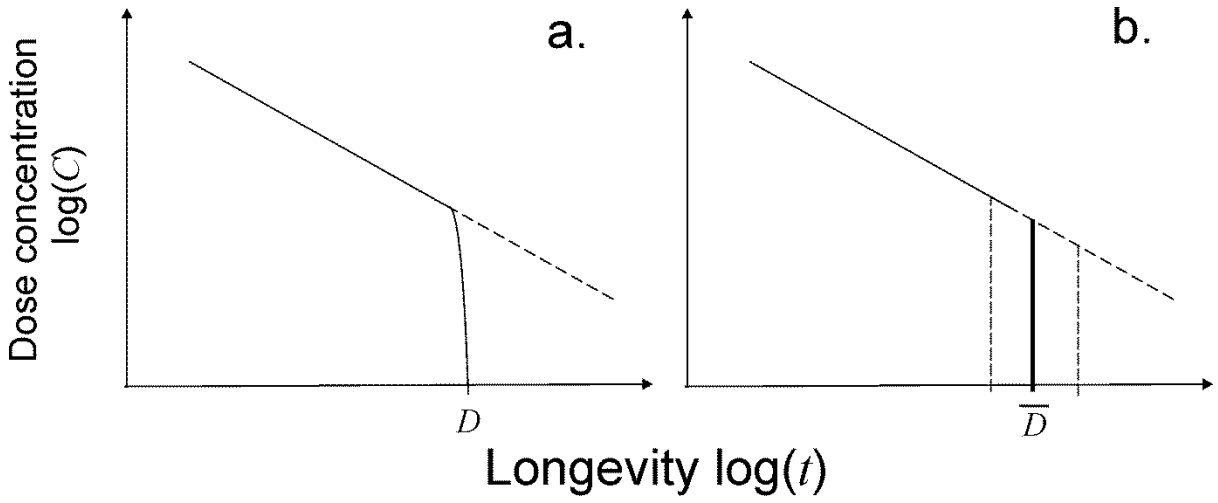

Fig. S2.7. ***Panel (a): The log(C)-vs.-log(t) relationship (solid line) is constrained at low doses by the organism's maximum longevity, D, which creates a 'hockey stick' shape; the dashed diagonal line indicates the theoretical (and unattainable) longevity predicted by*** $Ct^b = k$. ***Panel (b): the observed mean longevity of undosed bees,*** $\bar{D}$***, and its observed sampling variation (SD) is used to establish a 95% confidence interval (vertical dashed lines) on dose-independent longevity in individual cages.***

For example, Sánchez-Bayo (2009) states:

'Indeed, at a concentration of zero the time required to cause [an] effect on a group of organisms is theoretically infinite. … It is also evident that the life span of the organism determines the *upper limit of the curve*.' (Italics ours)

It is essential that the test for TRT is performed only on mortality data that describes toxic effects because mortality due to senescence also follows a pattern of 'time reinforcement' (i.e. the effects of old age intensify with time), which could confound the inference of TRT. Consequently, it is very important to carefully exclude mortality due to senescence, which otherwise biases the analysis to mistakenly detect time-reinforced toxicity, TRT. We therefore developed an objective protocol to circumscribe the appropriate subset of data. Importantly, the protocol itself did not require us to inspect the dose-vs.-longevity relationship for the appearance of TRT. Instead, we used the confidence interval on the longevity of the control (undosed) bees to objectively identify the *upper limit of the curve* referred to by Sánchez-Bayo *et al*. (Fig. S2.7b).

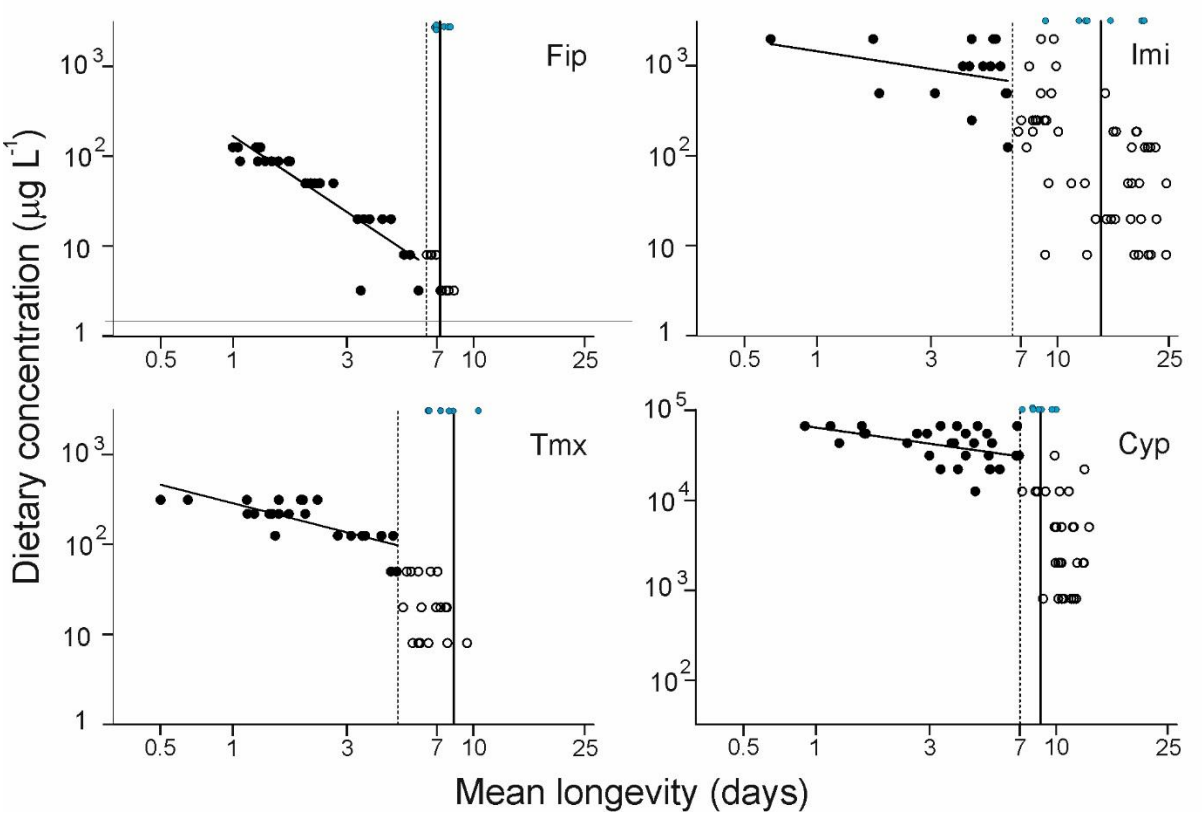

Figure S2.8. ***Identification of the dose-dependent range of C-vs-t relationships in four pesticides (Fip = fipronil; Imi = imidacloprid; Tmx = thiamethoxam; Cyp = cypermethrin).*** In each panel, the solid vertical line indicates the mean longevity of undosed controls, $\overline{D}$, and the dashed vertical line indicates its lower 95% confidence limit ($\overline{D}$ – 1.96SD). Open symbols indicate the mean longevity (days of exposure survived) in cages of dosed bees whose observed longevity fell in the confidence interval around $\overline{D}$. Black-filled symbols indicate cages of dosed bees whose value fell below the confidence interval for undosed bees, which indicated that the reduced longevity could be attributed to toxicity. Log(*C*)-*vs*-log(*t*) relationships between dietary concentration (*y*-axis: *C*, µg L^{-1}) and time-to-effect (*x*-axis: *t*, mean time until death of honey bees in an experimental cage) were fitted to the solid symbols (Main manuscript: Fig. 4). Blue-filled symbols indicate the mean longevity of undosed controls, which were used to estimate the confidence interval on the longevity in undosed cages (the *y*-axis position of these symbols can be disregarded.)

Specifically (Fig. S2.7b), we used the mean longevity of undosed bees, $\overline{D}$, and its observed sampling variation (SD) to establish a 95% confidence interval (vertical dashed

lines) on dose-independent longevity in *individual* cages (hence SE is not used). Observations of shorter longevity (i.e. below the lower CI on $\overline{D}$) in individual cages of dosed bees are reasonably considered dose-dependent. Therefore, we used this confidence limit to exclude data comprising the hockey-stick non-linearity and to thereby delineate the appropriate dose-dependent range in which to fit the straight-line log(*C*)-*vs.*-log(*t*) relationship (Fig. S2.8).

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Section S3: The demographic simulation of a honey bee colony and its parameterisation for environmentally relevant exposures

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 (Khoury, Myerscough & Barron 2011) and then perturbed the mortality rate according to the effects that we had quantified experimentally.

Methods

S3.1 Demographic simulation of the impact of mortality on a honey bee colony exposed to dietary pesticides

To explore 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 by additionally applying pesticide-dependent mortality (M_P) to hive bees (Fig. S3.1) and described the population dynamics of the control colony using previously determined parameter values (L = 2000, alpha = 0.25, theta = 0.75 (Khoury, Myerscough & Barron 2011); M_B = 0.154 (Henry *et al.* 2012a); w = 22000 (Henry *et al.* 2012b) 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 oilseed rape (Henry *et al.* 2012b).

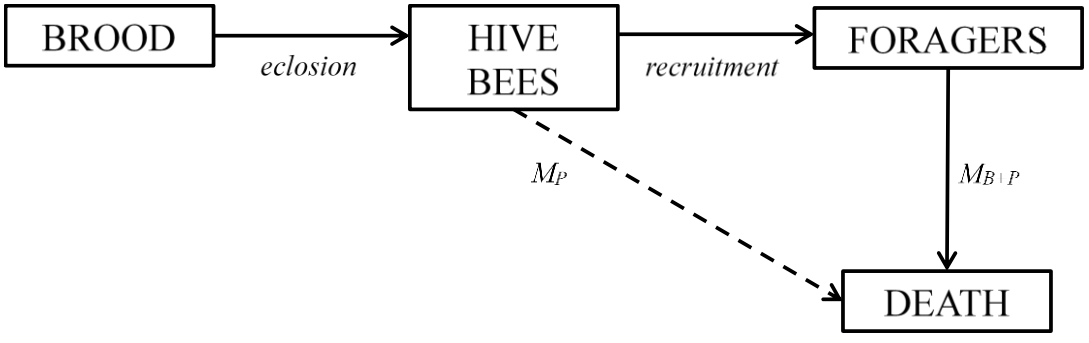

Figure S3.1. ***Schematic diagram of a demographic model of a honey bee colony modified from Khoury et al. (2011).*** The additional mortality added by our modification is indicated by a dashed line; M_P denotes the pesticide-dependent mortality rate and M_B denotes the 'base-line' pesticide-independent mortality rate.

Following Khoury *et al*. (2011), the rate of increase of the number of hive bees is determined by the number of brood and the rate of eclosion, given as $L\left(\frac{N}{w+N}\right)$ where L is the queen's laying rate, N is the total number of adult bees in the hive and w determines the rate at which the rate of eclosion approaches L as N increases. The rate at which hive bees are recruited as foragers is given by $H\left[\alpha-\sigma\left(\frac{F}{N}\right)\right]$, where α is the maximum rate of recruitment, σ is the rate of reversion of foragers back to hive bees, H is the number of hive bees and F is the number of foragers present in the colony.

The model of Khoury *et al.* was modified (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 (see S3.2).

S3.2 Estimation of per capita daily mortality rates at environmentally relevant doses

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 bees alive was three or more individuals. Specifically, for each dose j we calculated $\bar{p}_j = E_{i=1}^{n}(m_i/N_i)$, where m_i denotes the number of bees dying on the i^{th} day of the exposure, N_i denotes the number of bees alive at the beginning of day i, and $E_{i=1}^{n}$ indicates that the expected value is calculated across the interval from the first day of the exposure, $i = 1$, until the last day on which three of bees are alive, denoted n. Given our experimental design, the value of $\bar{p}_j$ therefore estimates the *population-wide* average mortality rate in a group of bees of mixed age and mixed duration of exposure, which is appropriate given the intermittent emergence times of newly-hatched adult bees. When *individual* bees experience TRT, this will elevate the values of m_i at the higher values of i, which will raise the value of $\bar{p}_j$ relative to that of the undosed control bees, as required.

We fitted $\bar{p}_j$-vs.-dose regressions in order to estimate M_{base} and M_{total}. Specifically, the fitted intercept estimates the daily mortality rate at zero dose, which is the background or 'baseline' mortality, denoted M_{base}. The value of the total daily mortality rate M_{total} is

found by solving the $\bar{p}_j$-vs.-dose regression at the environmentally relevant dose, $j = d$ (Fig. S3.2).

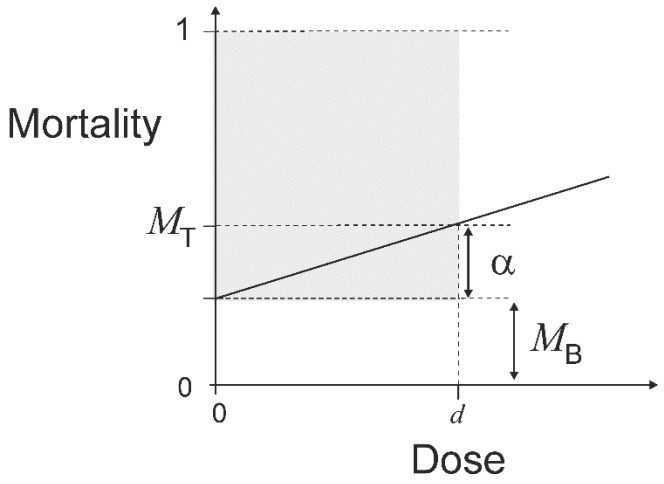

Fig. S3.2. ***The relationship between dose (x-axis) and per capita daily mortality rate (y-axis). The solid diagonal line indicates a hypothetical mortality-vs-dose relationship. The grey area indicates the proportion of bees (1 – M_{base}) that survive background mortality due to natural causes.***

Using M_{base} and M_{total} obtained from the above regressions, we then estimated the daily mortality rate due to each dietary pesticide at the environmentally realistic concentration, denoted $M_{pesticide}$, by adopting a probability-based framework as follows.

We first assume that pesticide-induced mortality applies to individual bees that survive the baseline mortality rate. I.e. mortality acts sequentially similarly to Abbott's correction:

$$M_{total} = M_{base} + (1 - M_{base})\, M_{pesticide} \qquad \text{Eq. S3.1}$$

By rearrangement of Eq. S3.1 we obtain:

$$M_{pesticide} = (M_{total} - M_{base})/(1 - M_{base}) \qquad \text{Eq. S3.2}$$

We then solve Eq. S3.2 for $M_{pesticide}$ after using the fitted dose-response (M_{total} *vs. dose*) relationships obtained previously to estimate M_{total} at an environmentally realistic dietary concentration of residues in nectar. The basis for this is shown in Fig. S3.1

In our demographic model we apply mortality sequentially (similarly to Abbott's correction), so that the dose-dependent proportion of bees dying above baseline mortality, α, is obtained by multiplying the death rate due to pesticides, M_P, by the probability of

surviving baseline mortality, which is given by $(1 - M_B)$ and indicated by the grey area in Fig. S3.2. So we obtain M_P by solving:

$(1 - M_B)\ M_P = \alpha$; and so $M_P = \alpha /(1 - M_B)$.

S3.3 Results

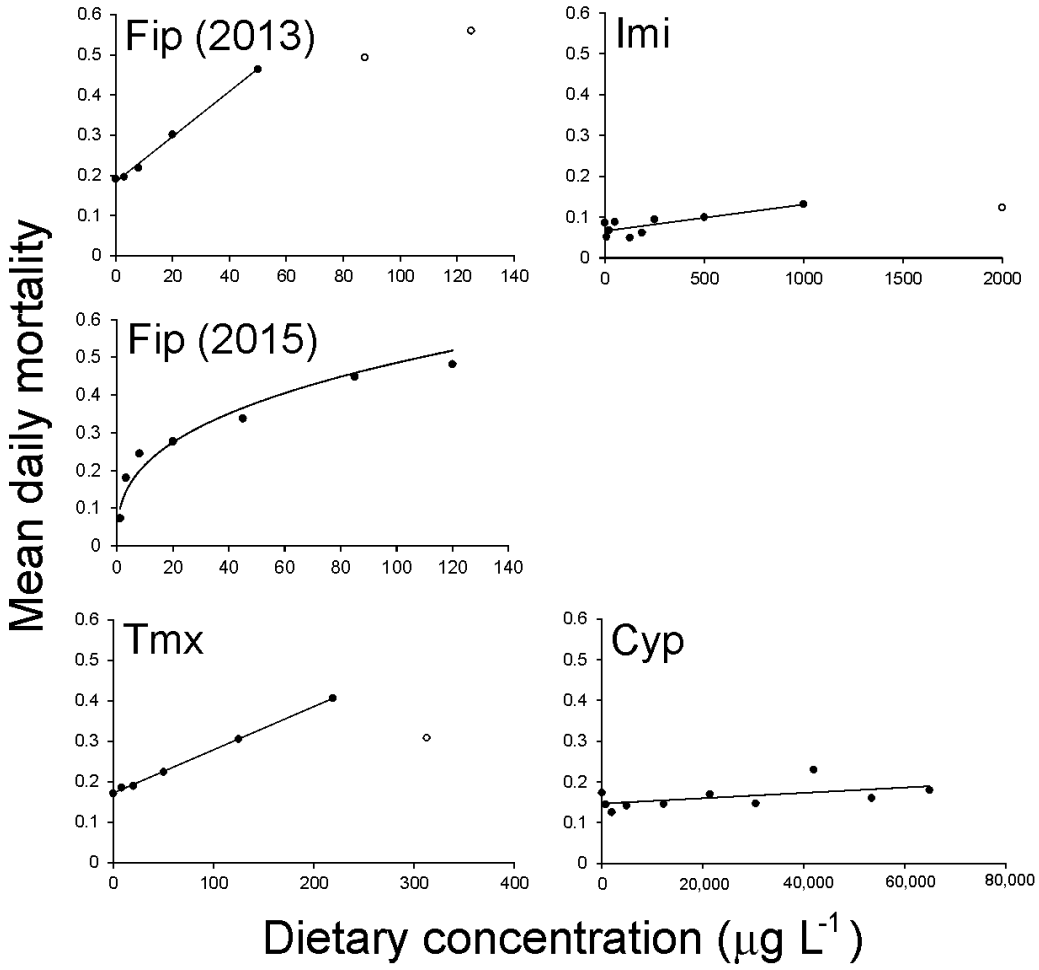

Figure S3.3. ***Dose-dependent variation in mean daily mortality rate of honey bee workers, $\bar{p}$.*** For each pesticide (Fip = fipronil - 2013 and 2015 experiments, Imi = imidacloprid, Tmx = thiamethoxam, Cyp = cypermethrin), the panels each show the mean daily mortality rate of honeybee workers (*y*-axis: mean daily mortality rate) exposed to various dietary concentrations of the pesticide (*x*-axis; toxicant concentration in dietary syrup in μg L^{-1}.) Each solid line indicates the fitted regression used to estimate the mortality rate at an environmentally relevant exposure. (Fip: 2013, $r^2 > 0.99$, 2015, $r^2 = 0.92$; Imi: $r^2 = 0.65$; Tmx: $r^2 > 0.99$; Cyp: $r^2 = 0.28$). Open symbols indicate data points not included in the regression analysis.

We fitted a least-squares linear relationship between the dietary concentration of pesticide (*dose*) and the daily mortality rate only in the linear region that spanned the environmentally realistic concentrations, for which we required a value of $\bar{p}$. Some data points at the highest doses did not belong to this relationship (e.g. the daily rate of mortality saturated) and were excluded. To obtain a good fit, we fitted a power relationship to the fipronil (2015) exposure.

Using these values, we fitted a least-squares relationship between the dietary concentration of pesticide (*dose*) and the daily *per capita* mortality rate, denoted M_{total} because this comprises both deaths due to the toxicant and also the background pesticide-independent death rate:

Fipronil:

2013: $M_{total} = 0.0056dose + 0.1833$; r-squared > 0.99;

2015: $M_{total} = 0.0944dose\text{^}0.356$; r-squared = 0.92;

Imidacloprid: $M_{total} = 0.0006dose + 0.0659$, r-squared = 0.65;

Thiamethoxam: $M_{total} = 0.0011dose + 0.1721$, r-squared > 0.99;

Cypermethrin: $M_{total} = 7\times10^{-7}dose + 0.1468$, r-squared = 0.28.

Using these fitted dose-response (i.e. M_{total} *vs*. *dose*) relationships, we estimated the pesticide-independent mortality rate, denoted M_{base}, from the intercept of each linear regression (i.e. the rate at zero-dose) and the pesticide-dependent mortality rate, denoted $M_{pesticide}$, by solving the equations at environmentally relevant doses (Table S3.1).

Toxicant	M_{base}	M_{total}	$M_{pesticide}$
Fipronil (2013)	0.1833	0.2197	0.0446
Fipronil (2015)	0.0944	0.1837	0.0986
Imidacloprid	0.0659	0.0698	0.0042
Thiamethoxam	0.1721	0.1793	0.0086
Cypermethrin	0.1468	0.1468	0.00001

Table S3.1. ***Estimates of parameters used in the demographic simulation*.**

References

Henry, M, Beguin, M, Requier, F, Rollin, O, Odoux, J-F, Aupinel, P, Aptel, J., Tchamitchian, S & Decourtye, A (2012a) A common pesticide decreases foraging success and survival in honey bees. *Science* 336:348-350.

Henry, M, Béguin, M, Requier, F, Rollin, O, Odoux, J-F, Aupinel, P, Aptel, J, Tchamitchian, S & Decourtye, A (2012b) Response to Comment on "A Common Pesticide Decreases Foraging Success and Survival in Honey Bees". *Science* 337:1453.

Khoury, DS, Myerscough, MR & Barron, AB (2011) A quantitative model of honey bee colony population dynamics. *PLoS ONE* 6:6.

Section S4: Survival during pesticide exposures

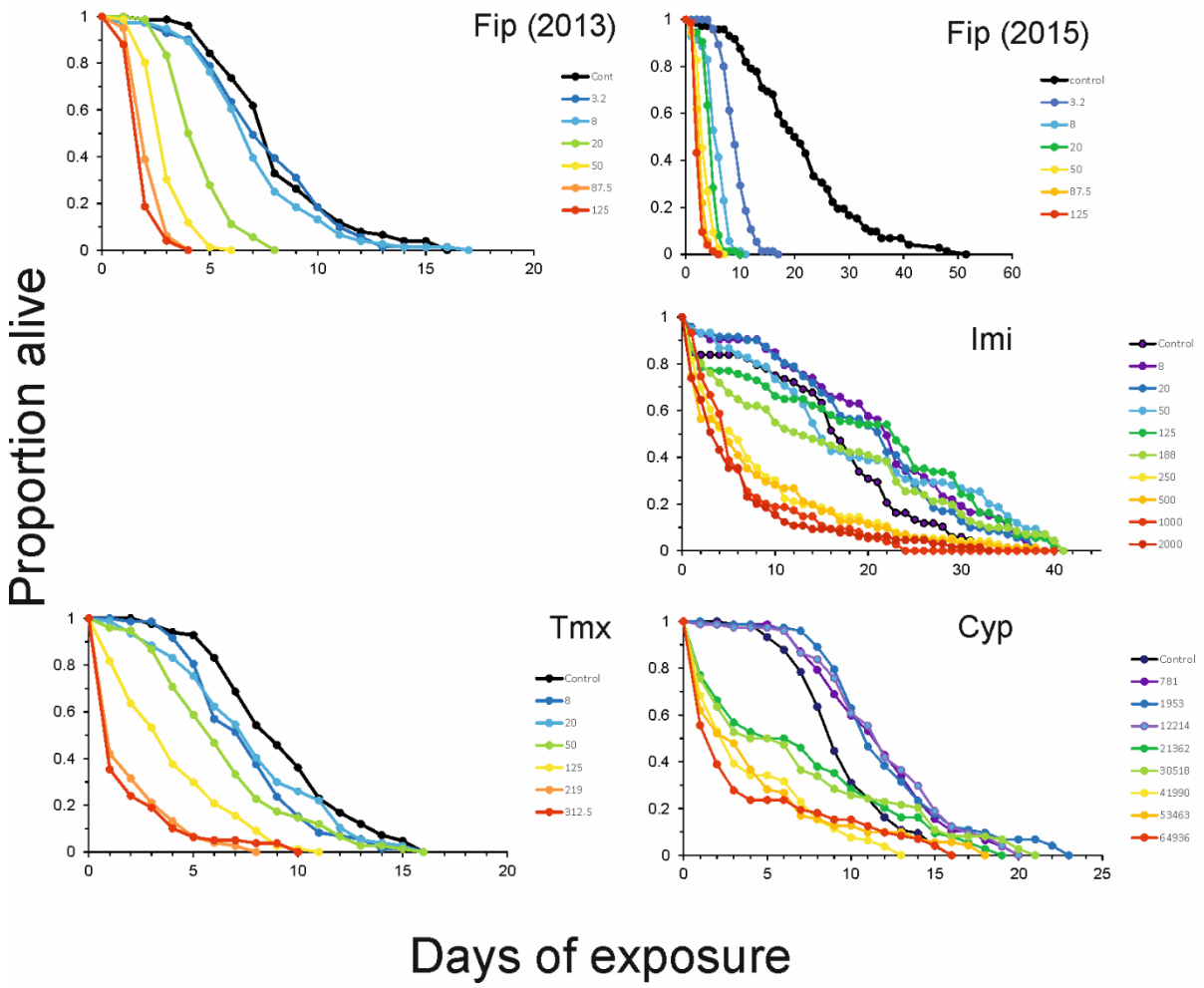

Fig. S4.1. Proportion of bees alive (*y*-axis) in relation to days of exposure (*x*-axis) in five separate experiments each involving a dietary pesticide: fipronil (Fip) in 2013 and 2015; imidacloprid (Imi); thiamethoxam (Tmx); and cypermethrin (Cyp). Doses are indicated by color: control = black; other doses indicated on legends (μg L^{-1}).

Section S5. Demographic outcomes for colonies exposed to imidacloprid or thiamethoxam

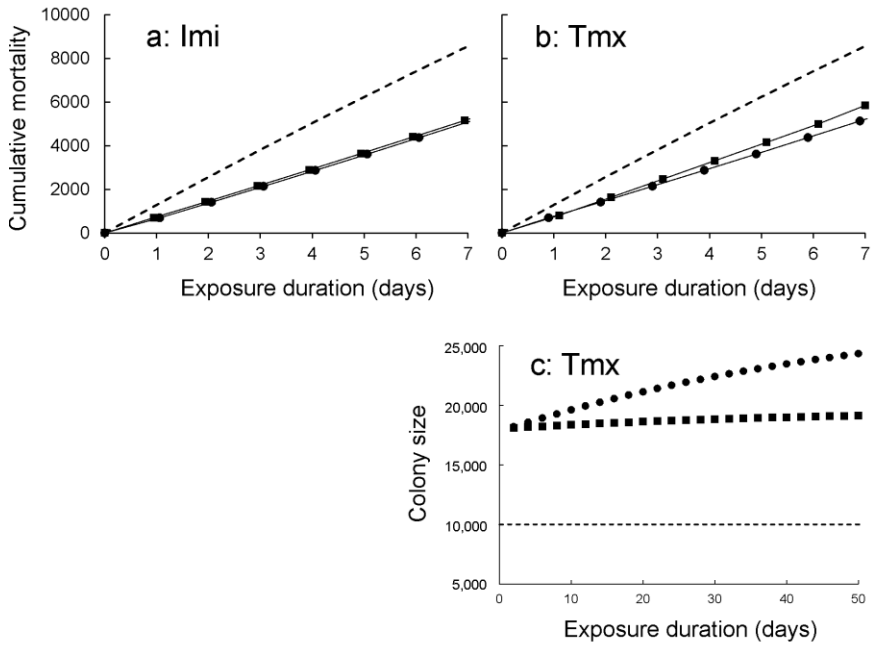

Figure S5.1. ***Colony performance during exposures to imidacloprid and thiamethoxam.*** Panel (a): Cumulative mortality (*y*-axis: total number of dead adult workers) over one week (*x*-axis: days) for a control colony (filled circles) vs. a colony exposed to either dietary imidacloprid (square symbols) or fipronil (dashed line). Panel (b): Cumulative mortality over one week for a control colony (filled circles) vs. a colony exposed to either dietary thiamethoxam (square symbols) or fipronil (dashed line). Note that these graphs depict total mortality and not mortality at the hive entrance (c.f. Fig. 2 of the main paper) because the purpose of (a) and (b) is to show the similarity between levels of overall mortality in control colonies and colonies exposed to either imidacloprid or thiamethoxam. (Some symbols are displaced slightly in the *x*-plane for clarity.)

Lower panel (c): Number of live adult bees (*y*-axis) in relation to time (*x*-axis, days of exposure) for a colony exposed to thiamethoxam (square symbols) or an unexposed control (round symbols). Exposure to cypermethrin had only a negligible impact and results are not shown.

Section S6: signatures of TRT in thiamethoxam and cypermethrin

Thiamethoxam exhibited neither of the signatures of TRT (*C*-*vs*.-*t* relationship, regression analysis, $b = 0.7 \pm 0.13$, Fig. S6.1a; ingestion-*vs*.-longevity relationship: correlation analysis, Spearman's *rho* = 0.04, P >0.05; Fig. S6.1c).
Cypermethrin exhibited neither of the signatures of TRT (*C*-*vs*.-*t* relationship, regression analysis, $b = 0.4 \pm 0.13$; Fig. S6.1b; ingestion-*vs*.-longevity relationship: correlation analysis, Spearman's *rho* = 0.62, P = <0.001; Fig. S6.1d).

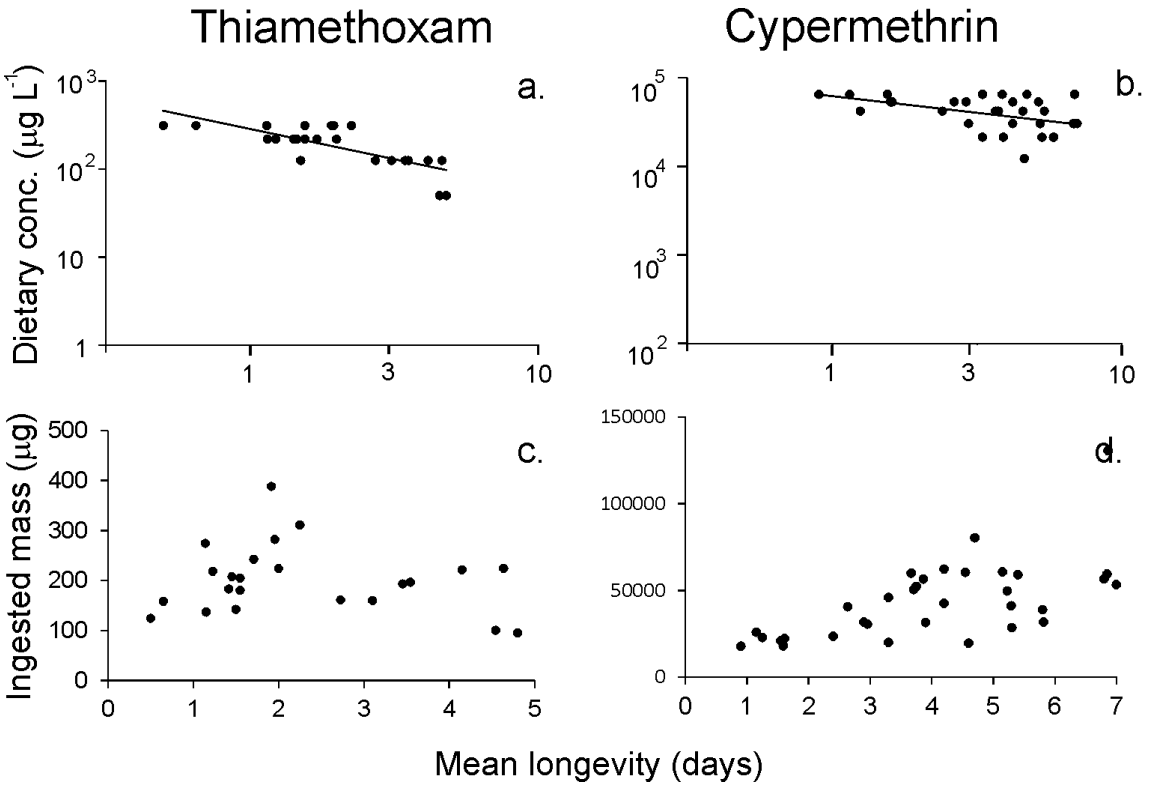

Fig. S6.1. ***TRT indicators for thiamethoxam and cypermethrin*****.** Panels a and b: evaluation for time-reinforced toxicity by *C*-*vs*-*t* relationships. Each filled symbol indicates the mean longevity (days of exposure survived) in a cage of dosed bees whose value fell below the confidence interval for undosed bees, which indicated that the reduced longevity could be attributed to toxicity. Log(*C*)-*vs*-log(*t*) relationships between dietary concentration (*y*-axis: *C*, µg L^{-1}) and time-to-effect (*x*-axis: *t*, mean time until death of honey bees in an experimental cage) were fitted to the solid symbols. Panels c and d: evaluation by ingestion-*vs*-longevity relationships. Each

filled symbol represents a single cage of honey bees based on: the total mass of toxicant consumed by the bees before their deaths (*y*-axis: mass ingested, ng) and the mean longevity of the exposed bees (*x*-axis: mean days of exposure survived). Neither produces a significant negative trend (Spearman correlation analysis, $P > 0.05$). These data originate from cages of dosed bees whose observed longevity is below the lower confidence interval for longevity in undosed bees.

Section 7. Evaluation of Abbink's claim of irreversible ligand-receptor binding in imidacloprid

Several previous papers (Tennekes 2010, 2017; Tennekes & Sánchez-Bayo 2013) claim that a radio-ligand binding experiment that was performed on stable flies, *Stomoxys calcitrans* by Abbink (1991), exhibited irreversible binding by imidacloprid.
Below, we demonstrate that Abbink (1991) offers no experimental evidence for irreversible ligand-receptor binding by imidacloprid (in fact, the data show the exact opposite).
Abbink (Abbink 1991) conducted radioligand binding experiments on homogenised fly heads of stable flies (*Stomoxys calcitrans*). N-[propionyl-^{3}H] propionated alpha-bungarotoxin was used as the radioligand (RL). Competition experiments were conducted using non-radiolabelled ligands (NRLs), nicotine and imidacloprid.
Abbink determined the '50% inhibition concentration' (IC_{50}) of each NRL by conducting incubations of homogenised nervous tissue, the RL and various concentrations of NRL. The results are shown in Fig. S7.1.

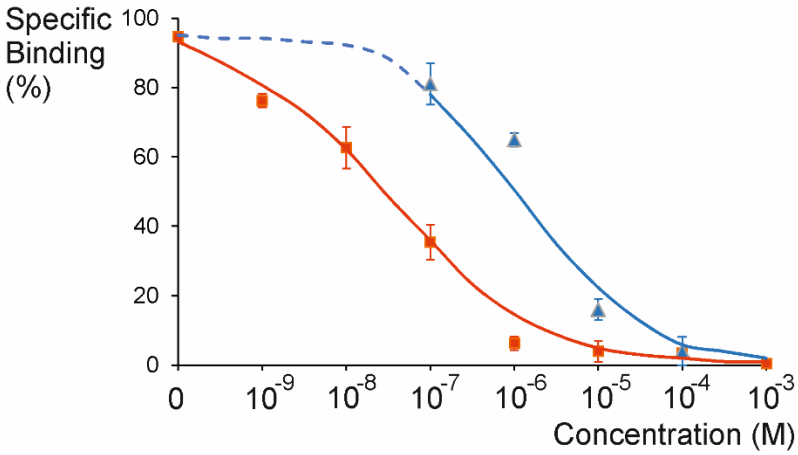

Fig. S7.1. ***Concentration-dependent binding affinity of nicotine (red symbols) and imidacloprid (blue symbols): results of competitive displacement experiments involving RL-alpha-bungarotoxin and a homogenate of fly heads*** **(*Stomoxys calcitrans*).** Digitized from Fig. 4 in Abbink (1991). The *y*-axis evaluates the proportion of receptors occupied by the radioligand (∝-bungarotoxin) as a function of the (*x*-axis) concentration of the competitor non-radioligand

(nicotine or imidacloprid) that was initially incubated with the nervous tissue, i.e. before the introduction of the radioligand.

Abbink states that the NRL was added *first* to the homogenate (p 187): 'Aliquots (250 μL) of homogenate were diluted with 700 μL incubation buffer (50 mM TRIS/HCL pH 7.4, 160 mM NaCl, 2 mg/mL bovine serum albumin) and nicotine (final concentration 0.001 to 1000 μM) or imidacloprid or its derivative or analogues (final concentration 0.1 to 100 μM. … *After* preliminary incubation at 26°C for 30 min, 50 μL radiolabelled alpha-bungarotoxin solution was added.' [Italics ours]
Therefore, Fig S7.1 describes the extent to which alpha-bungarotoxin displaces the NRL. I.e. the experiment demonstrates dissociation (reversibility) of ligand-receptor binding by the NRLs (nicotine and imidacloprid), which is the opposite of the many previously published claims about Abbink's findings.

References

Abbink, J (1991) The biochemistry of imidacloprid. *Pflanzenschutz-Nachrichten Bayer* 44:183-195.

Tennekes, H (2017) Dose: Time-to-effect analyses can identify hazardous chemicals at an early stage of product development. *Environ. Risk Asses.* 1:65-70.

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Section S8. Evaluation of a previous claim that imidacloprid causes TRT in honey bees

S8.1 Overview:- Here, we re-examine three log(*C*)-*vs.*-log(*t*) relationships presented by Rondeau *et al*. (2014), which were said by the authors to show that imidacloprid causes time-reinforced toxicity in honey bees.

Rondeau *et al*. analysed three datasets that comprised timescales of mortality in adult honey bees that were subjected to 10-day exposures of various dietary concentrations of imidacloprid.

We reject two of the datasets as unsuitable and we re-analyse the third dataset and find no evidence that imidacloprid caused symptoms of time-reinforced toxicity (TRT). Specifically, our main conclusion is depicted in Fig. S8.1.

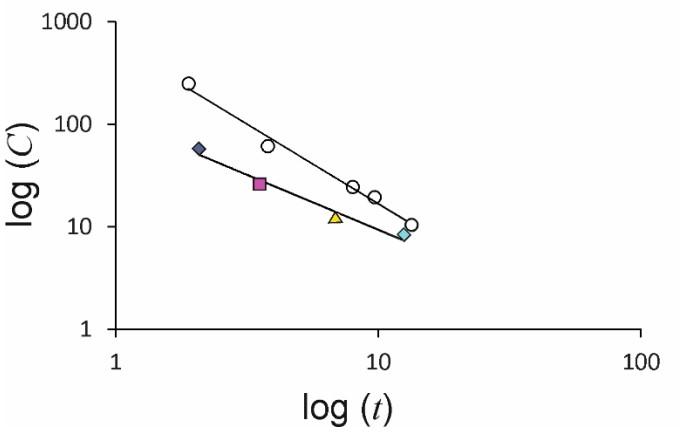

Fig. S8.1. ***Log(C)-vs.-log(t) relationships obtained from the Defra (2007) dataset according to Rondeau et al. (open symbols) and our re-analysis (coloured symbols).*** The regression lines have slopes of b = -1.6 according to Rondeau *et al*., and b = -1.1, which is the result of our re-analysis.

We describe the reasoning for our conclusion below.

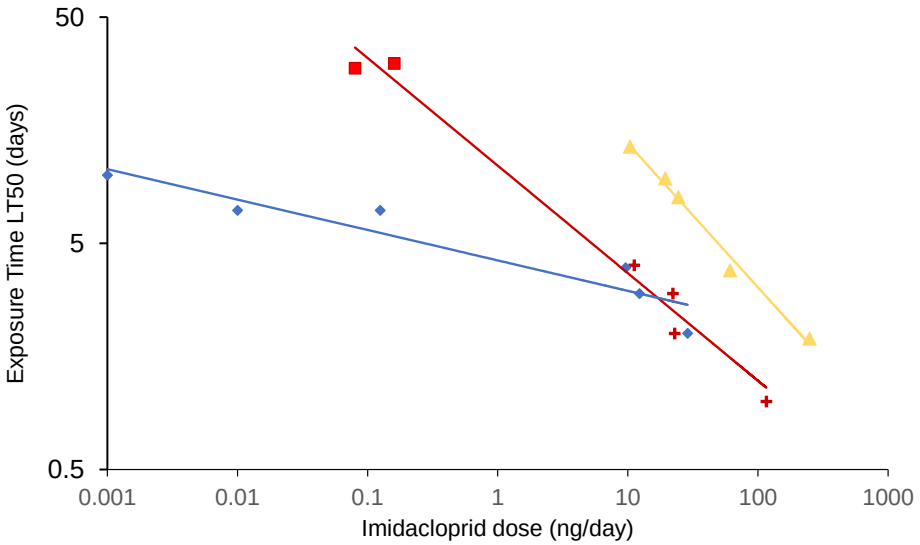

Fig. S8.2. ***Figure 2 in Rondeau et al., which depicts log(t)-vs.-log(C) relationships from previously published sources.*** Blue diamonds: data from Suchail *et al*. (2001). Red symbols: data from Dechaume-Moncharmont et al. (2003) (squares) and 'many researchers average' (crosses). Yellow triangles: data from Defra (2007).

We reject two of the datasets (blue, red symbols) as unsuitable with the following reasoning.

S8.2 Suchail et al. (2001) is an anomaly

We do not consider further the relationship based on data published by Suchail *et al*. (2001) (blue symbols), because the dose-dependence is anomalous. Specifically, no other published study has reported that dietary imidacloprid at such low doses causes such a high level of mortality (Fig. S8.3).

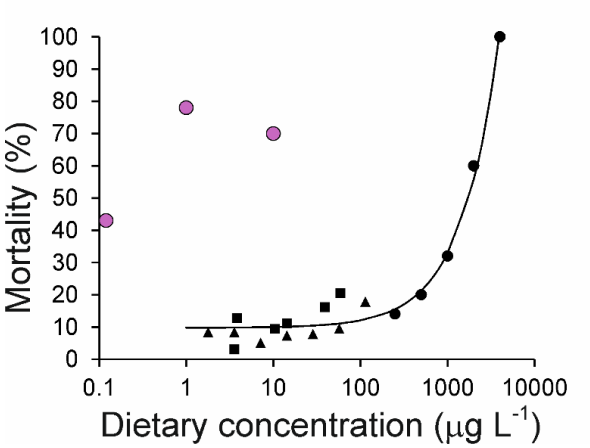

Fig. S8.3. Suchail *et al*. (2001) reported anomalous results (●) when compared to the consensus dose-dependence of mortality (symbols: ● Defra (2007); ■ Decourtye et al. (2003) winter bees; ▲ Decourtye et al. (2003) summer bees). *Y*-axis: proportion of honey bees dead after 10-day exposure; *x*-axis: concentration of imidacloprid in feeder syrup; μg L^{-1}.

S8.3 Artefacts in Haber exponents can arise by pooling datasets

Also, we do not consider further the relationship based on data derived from a compilation of separate studies (red symbols) for two reasons. First, combining data points across studies without overlapping dose-response relationships (that thereby demonstrate consistency in responses to doses) makes the procedure of pooling *different* doses from separate studies intrinsically insecure. The observed levels of variability in dose-sensitivity among different experiments is very great (e.g. 'the oral LD50 value of imidacloprid may vary widely - factor 20 - in the honey bee') (Decourtye & Devillers 2010), probably because of differences in the bees used (e.g. genetic strain, health status, etc.). Specifically, the existence of uncontrolled variation in dose-sensitivity among studies (as, for example, indicated by the different positions of red and yellow symbols in Fig. S8.2) implies that dose-dependence should be evaluated only in demonstrably consistent log(*t*)-*vs.*-log(*C*) relationships, such as those that are evaluated across a statistically coherent population of honey bees. Since LD_{50} varies widely among studies, using a single datum from a single study for each *C*-*vs.*-*t* combination (as in Rondeau *et al*.) means that the slope (Haber exponent) of a four-point trend could be strongly

affected by the uncontrolled variation among studies in dose-sensitivity. In essence, the procedure of analysing different doses from a small number of separate studies is intrinsically insecure. A meta-analysis to estimate the Haber exponent from a fitted C-vs-t relationship can only be done robustly where several studies replicate (approximately) each of the test concentrations.

Second, Rondeau *et al*. did not publish the data underlying points labelled as 'many researcher averages', so the uncertainty associated with these points cannot be statistically evaluated.

Therefore, we focus on the analysis of the data from the third study, namely that presented in the Defra report on systemic insecticides (DEFRA 2007), which we refer to below as the 'Defra dataset'.

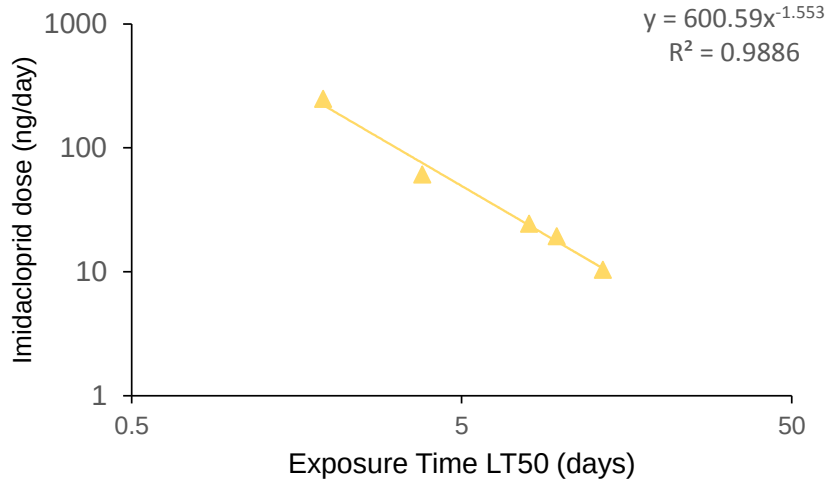

Fig S8.4. ***Log(C)-vs.-log(t) relationship obtained by using the values reported by Rondeau et al. from the Defra dataset, but plotted with the axes arranged as in our study.*** The fitted least-squares regression line has a slope of b = -1.6, which is evidence of TRT.

S8.4 Re-analysis of the Defra dataset

On the basis of their analysis of the Defra dataset, Rondeau *et al*. claimed that imidacloprid caused time-reinforced toxicity. Based on the values reported in their paper and plotting the relationship with the axes arranged as in our study [Fig S8.4:- y-axis: log(C); x-axis: log(t)], we would agree, because the slope of the log(C)-*vs.*-log(t) relationship is: b = -1.6.

We therefore re-analysed the Defra dataset to verify this result.

The Defra dataset describes mortality (percentage fatality among ten bees in an experimental cage) during a 10-day dietary exposure (Fig. S8.5).

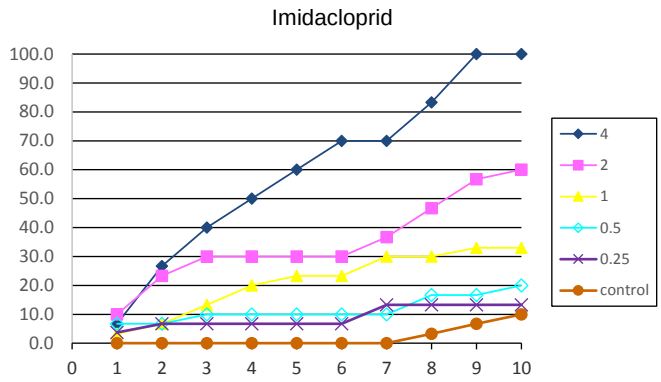

Fig. S8.5 ***Dose-dependence of mortality-exposure duration relationships in the Defra dataset*** [*x*-axis: days of exposure; *y*-axis: mortality (%) in cages of 10 individual bees.] Each linked series of symbols relates to a dietary concentration of imidacloprid; dietary concentrations shown in the inset legend are in units of μg/ml.

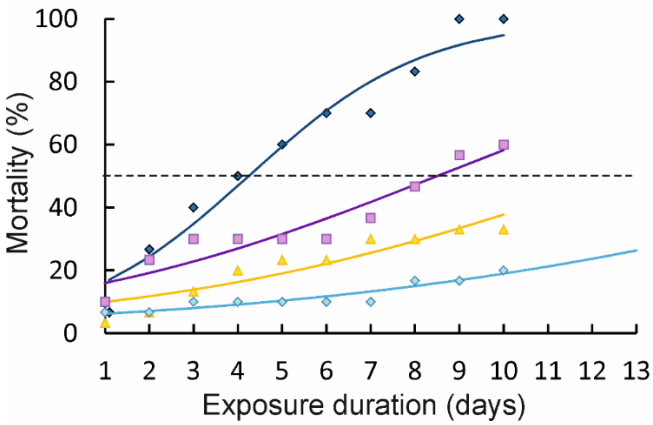

Fig. S8.6. ***Dose-dependence of mortality-duration relationships in the Defra dataset with fitted least-squares mortality-duration relationships.*** Doses are: 4000 μg L^{-1} (dark blue); 2000 μg L^{-1} (purple); 1000 μg L^{-1} (yellow); 500 μg L^{-1} (light blue).

Only two dose treatments attained 50% mortality of the caged bees during the 10-day exposure period and it requires extrapolation beyond the range of the available data to estimate the 'time to 50% fatality', or LT_{50}, of other doses (Fig. S8.6). We believe that LT_{50} could be reliably estimated in only the two highest doses and perhaps also the third

by extrapolation. Unlike Rondeau *et al*., we therefore do not use LT_{50} as the endpoint for evaluating the log(*t*)-*vs*.-log(*C*) relationship.

The log(*t*)-*vs*.-log(*C*) relationship can be evaluated for any endpoint; it is not restricted to the LT_{50}. Therefore, we evaluated the relationship for LT_{25}, or 'time to 25% fatality', which is the duration of exposure required to cause 25% mortality at a given dose (Fig. S8.7).

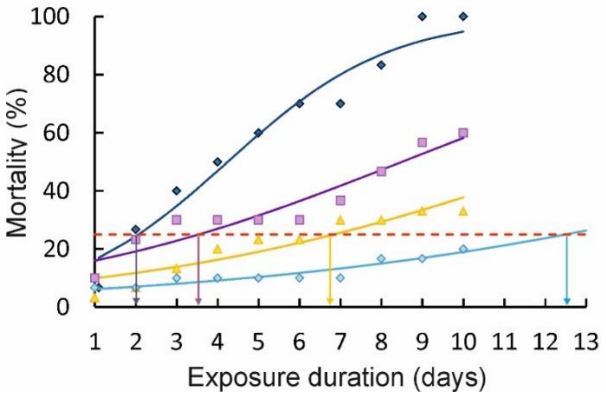

Fig. S8.7. ***Estimating the dose-dependence of LT_{25} in the Defra dataset from fitted least-squares mortality-duration relationships.***

Based on the dose-dependent mortality, we estimate LT_{25} for each of the four doses according to increasing dose as: 500 µg L^{-1} (light blue), 12.51; 1000 µg L^{-1} (yellow), 6.85; 2000 µg L^{-1} (purple), 3.55; 4000 µg L^{-1} (dark blue) 2.07 days (Fig. S8.7).

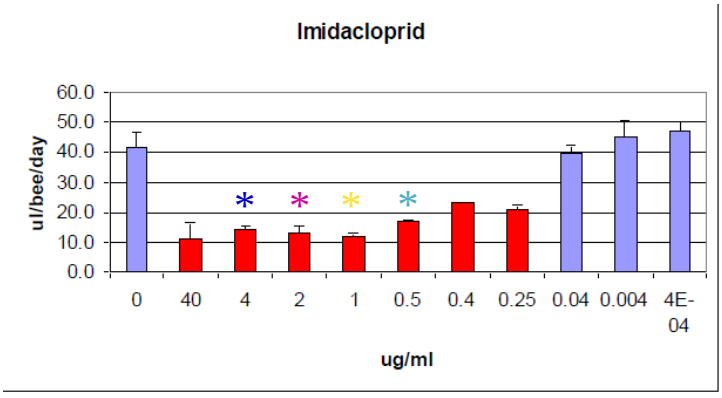

Fig. S8.8. ***Dose-dependence of syrup consumption (µL bee^{-1} d^{-1}) in the Defra dataset.***
Corresponding with the preceding mortality curves, the coloured asterisks identify relevant doses as: 4000 µg L^{-1} (dark blue); 2000 µg L^{-1} (purple); 1000 µg L^{-1} (yellow); 500 µg L^{-1} (light blue).

As a proxy for *C* in the log(*t*)-*vs.*-log(*C*) relationship, Rondeau *et al*. used the estimated daily *per capita* consumption of imidacloprid, which we obtained from the data on dose-dependent syrup consumption given in the Defra dataset.
Using the daily consumption rates at each dose (Fig. S8.8), we estimated the *per capita* daily consumption of imidacloprid in ng day^{-1} (Table S8.1).

µg L^{-1}	**µL day^{-1}**	**ng µL^{-1}**	**ng day^{-1}**
4000	14.3	4.0	57.2
2000	12.9	2.0	25.8
1000	11.9	1.0	11.9
500	16.9	0.5	8.5

Table S8.1. Derivation of dose-dependent daily per capita rates of imidacloprid ingestion by exposed honey bees. We note that these values do not match the series of values used in Fig. 2 of Rondeau *et al.* (Fig. S8.2), which were (according to increasing dose): 10.4; 19.4; 24.4; 61.0; 248.5 ng bee^{-1} d^{-1}.

Using the data on dose (*per capita* daily intake, Table S8.1) and exposure duration (LT_{25}, Fig. S8.7), we evaluated the slope of the log(*C*)-*vs.*-log(*t*) relationship, which is *b* = -1.1 (Fig. S8.1), which is consistent with the absence of TRT (see main text).

References

Decourtye, A & Devillers, J (2010) Ecotoxicity of neonicotinoid insecticides to bees. *Adv. Exp. Med. Biol.* 683:85-95.

Decourtye, A, Lacassie, E & Pham-Delegue, MH (2003) Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season. *Pest Manag. Sci.* 59:269-278.

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Rondeau, G, Sánchez-Bayo, F, Tennekes, HA, Decourtye, A, Ramírez-Romero, R & Desneux, N (2014) Delayed and time-cumulative toxicity of imidacloprid in bees, ants and termites. *Sci. Rep.* 4:5566.

Suchail, S, Guez, D & Belzunces, LP (2001) Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in *Apis mellifera*. *Environ. Toxicol. Chem.* 20:2482-2486.

Section S9: Comparison of dose-dependent mortality in the present and previous studies.

Our results (◆) exhibit higher levels of mortality at any given dose than observed in previous studies (Fig. S9.1), probably because the mixed-age cohorts that we studied contain older, frail individuals which are absent among the newly emerged bees normally used elsewhere. In the only other case where mixed-age cohorts were sampled directly from a colony (●), the results match ours more closely.

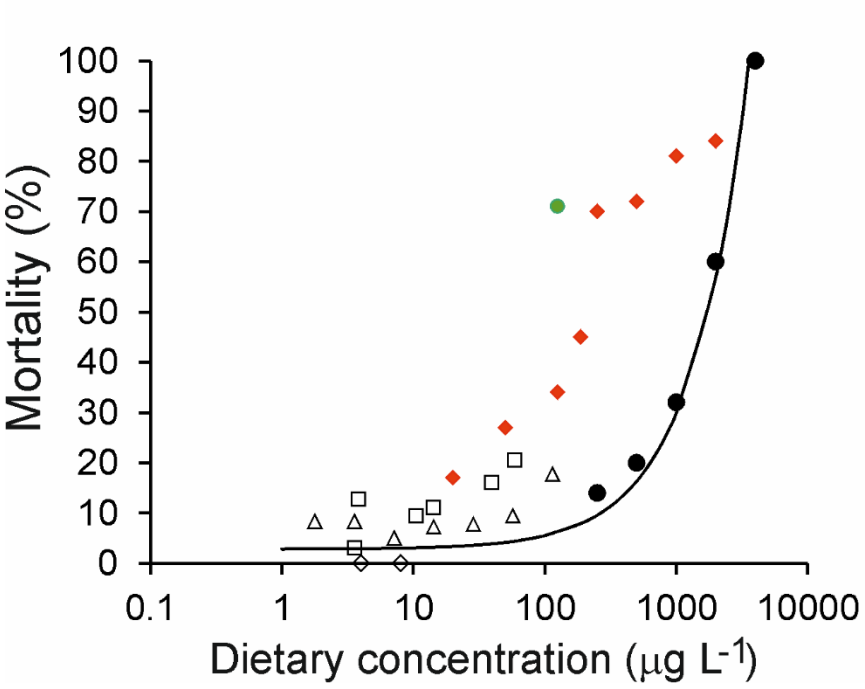

Fig. S9.1. ***Dose-dependent mortality during sustained (10 day) exposure to dietary imidacloprid compared between the present and previously published studies.*** *Y*-axis: proportion of honey bees dead after 10-day exposure in relation to concentration of imidacloprid in feeder syrup (*x*-axis: μg L^{-1}). Symbols: ◆ present study; ● (Sánchez-Bayo, Belzunces & Bonmatin 2017); ● Defra (2007); Decourtye et al. (2003): □ = winter bees, △ = summer bees; ◇ Dechaume-Moncharmont et al. 2003. Data are adjusted for control mortality by Abbott's correction. References in Section S1.

Section S10: Thermogenic activity by honey bees under laboratory conditions

For a non-thermogenic insect, the relatively low temperature of our laboratory environment compared to in-hive conditions (i.e. c. 25 °C vs. 33 °C, respectively) would be a cause of concern because the impact of toxicants can be strongly temperature-dependent even across a fairly small range. Honey bees, however, are capable of non-flight thermogenesis (NFT) and we find that they maintain their body temperatures above 30 °C in our standard laboratory cages for at least 24 h (Fig. S10.1). Consequently, it appears likely that metabolic processes of the bees in our laboratory experiments are functioning at approximately normal temperature and rates. Honey bee workers are likely often to engage in non-flight thermogenesis to incubate brood under normal in-hive conditions, so NFT-related activity in laboratory bees is not obviously anomalous.

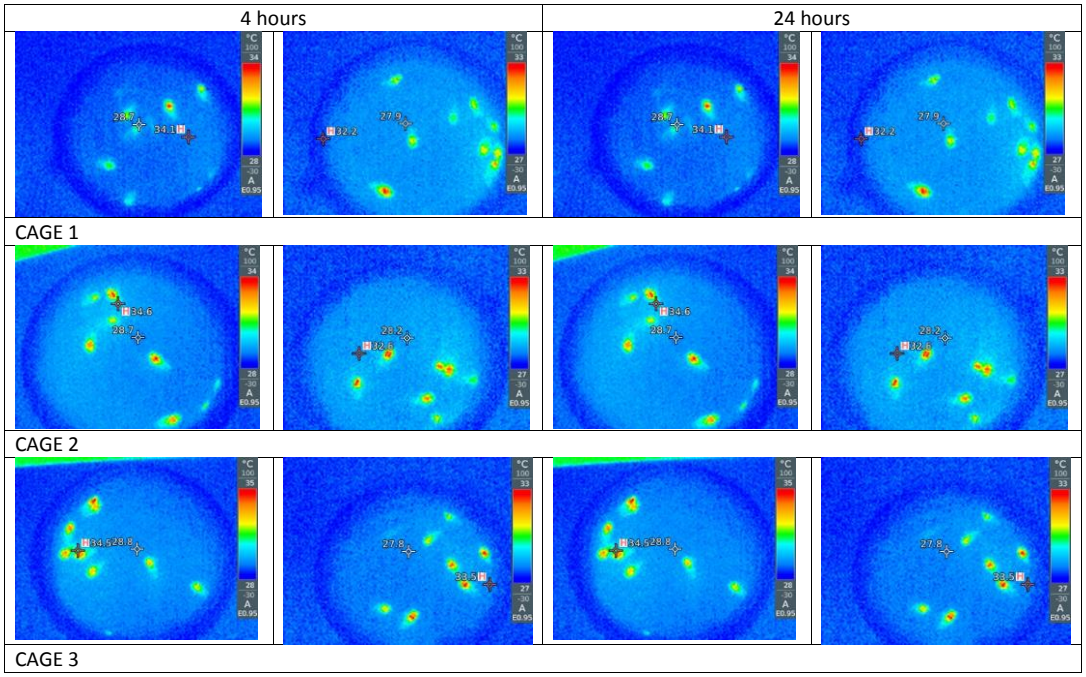

Fig. S10.1. Thermal images of summer honey bees in cages as used in our study in the CTR (controlled-temperature room) of our laboratory. Each of the three cages contained 10 adult worker honey bees taken from one of the apiaries used in our original study (only the subset of individuals walking on the mesh top was captured in each image.) Each red area indicates the thorax of a honey bee, which was in the range of 33 – 35 °C. Images were taken with a Testo 868 camera (Testo SE & Co., Lenzkirch, Germany) on 6th and 7th July, 2018.

If the dietary toxicants that we studied detrimentally reduced NFT, then falling to an ambient temperature below 31-33 °C (normal in-hive conditions) would be a realistic in-hive outcome whose occurrence is also supported by our laboratory set-up.

Section S11: Feeding (daily syrup consumption) of honey bees during exposure to dietary pesticides

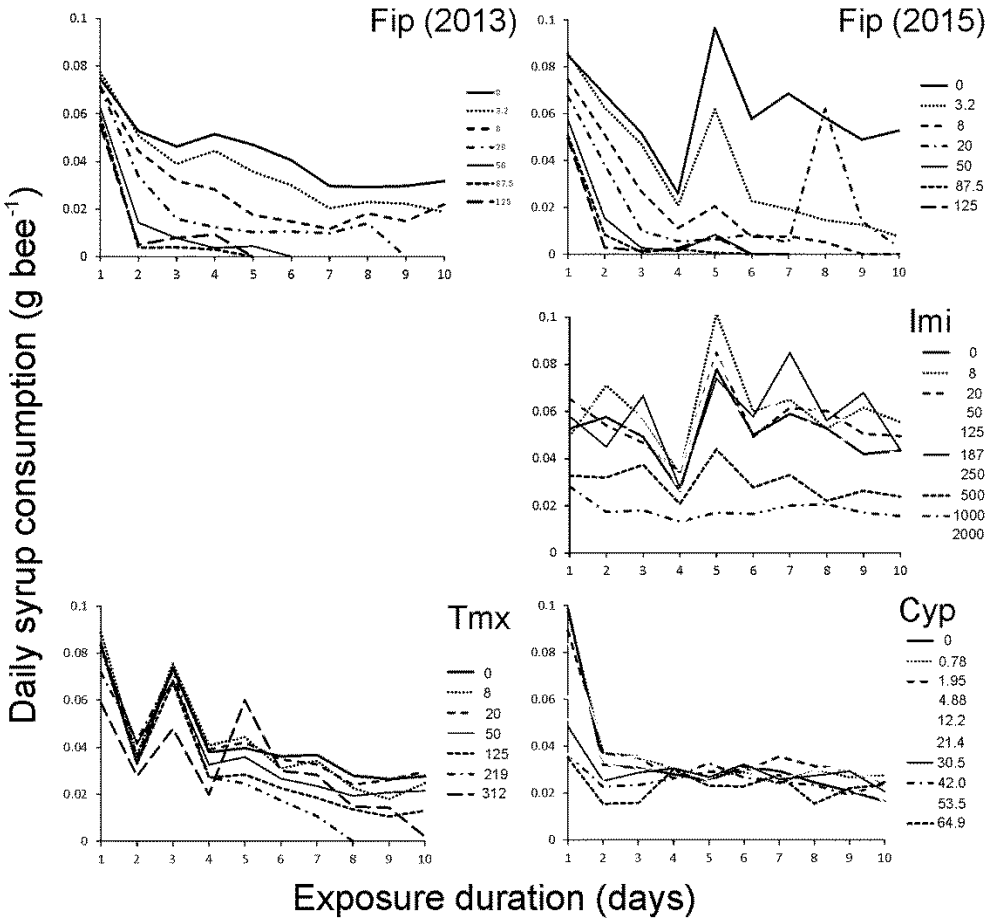

Figure S11.1. ***Effects of four pesticides on honeybee syrup consumption over time.*** Relationship between syrup consumption of honeybees at various pesticide doses (*y*-axis: mean daily syrup consumption per bees in g) and exposure time (*x*-axis: duration of exposure in days) for honeybees exposed to: (Fip) fipronil in 2013 and 2015 (0 to 125 µg L^{-1}, N = 7); (Imi) imidacloprid (0 to 2000 µg L^{-1}, N = 7); (Tmx) thiamethoxam (0 to 312.5 µg L^{-1}, N = 7); and (Cyp) cypermethrin (0 to 64.94 mg L^{-1}, N = 7).

The spikes in consumption that appear most prominently in 2015 experiments (fipronil and imidacloprid) coincide with variation in the time of day when feeders were changed. For example, on Days 4 and 5 of the 2015 experiments, feeders were weighed at approximately 10.00 and 16.00 respectively, which means that the consumption allocated to Day 5 in actuality arose over a 30 hour period.

Section S12: Whole-body residue analysis over time of honeybees exposed to fipronil at exact sampling points

Methods

Fipronil was obtained as a powder (analytical standard, PESTANAL®, Sigma Aldrich Co. LLC; product code: 46451) and was dissolved in acetone to form a stock solution of 2.9 µg ml-1, before being mixed with 50% w/v aqueous sugar solution (Attraker: 1.27 kg L-1 fructose/glucose/saccharose solution; Koppert B.V., Berkel en Rodenrijs, Netherlands) to produce the final dose used.

Adult worker honey bees of varied age were collected from managed colonies in Devon, UK. Honey bees were starved for two hours prior to dosing and were then briefly chilled to inactivity before being placed into cages in batches of 10 (plastic cage dimensions: 0.10 m diameter x 0.04 m height). Bees were maintained under controlled laboratory conditions (daily mean temperature = 24.4 °C ± 0.18 S.E.; mean relative humidity = 35.9 % ± 0.76 S.E.; 12:12 hours of low-light:darkness) and each cage of 10 bees was 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). This dose was chosen because it produces little mortality during a 6-day exposure but with quantifiable residues in body tissues. After this initial dose had been consumed (time 0), bees were provided with control (undosed) syrup thereafter. Cages were sampled at the time points of 0, 0.5, 1, 1.5, 2, 4 and 6 days post-dose and frozen at -20 °C. Control honey bees (no fipronil exposure) were collected after 0, 1, 2 and 4 days. Replication of each treatment (dosed, control) was n = 2 or n = 3 cages per time point. For purposes of display (Fig. 4), data from the two half-day time points (day 0.5, day 1.5; Fig. S12.1) were combined with the previous whole-day measures (e.g. day 0.5 with day 0) to estimate the mean residue level across each day.

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) (Section S13). Bees fed control syrup were analysed only for residues of fipronil sulfone.

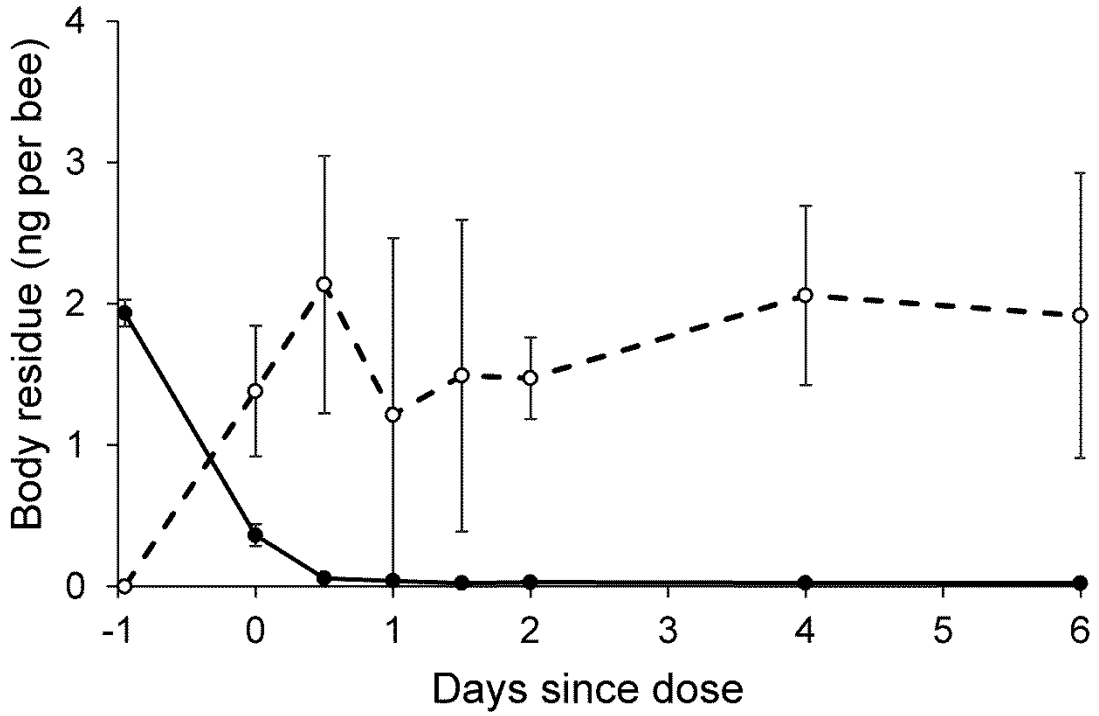

Figure S12.1. Whole-body residue analysis over time of honeybees exposed to fipronil at exact sampling points. Body residues (*y*-axis: mean ng bee^{-1}) were measured at intervals over a six day period (*x*-axis: days since dose) after a single acute dietary exposure to fipronil in syrup at 145 µg L^{-1}. Day = 0 indicates samples collected immediately after dosing and Day = -1 indicates the estimated initial ingestion of fipronil. Error bars denote ± 1 SE. Concentrations in undosed bees were less than 0.02 ng bee^{-1} fipronil and 0.11 ng bee^{-1} fipronil sulfone. Note: some small error bars are obscured by data points. Mean residues are connected for ease of inspection only.

Section S13: Selected-ion monitoring (SIM) parameters used for GC-MS analysis.

	Target		**Q1**		**Q2**	
Analyte	**m/z**	**dwell**	**m/z**	**dwell**	**m/z**	**dwell**
Fipronil	367	160	351	140	369	140
Fipronil sulfone	383	40	385	40	255	40

Table S13.1.