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WINGS AS A NEW ROUTE OF EXPOSURE TO PESTICIDES IN THE HONEY BEE

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Abstract: In pesticide risk assessment, estimating the routes and levels of exposure is critical. For honey bees subjected to pesticide spray, toxicity is assessed by thorax contact to account for all possible contact exposures. In the present study, the authors tested 6 active substances with different hydrophobicity. For the first time, the authors demonstrated that it is possible to induce mortality by pesticide contact with only the wings of the honey bee. The toxicities induced by contact with the wings and thorax were similar, with the wing median lethal dose (LD50) being 0.99 to 2.23 times higher than that of the thorax. This finding demonstrates that the wings represent a relevant route of exposure in the honey bee. In a second approach, the authors estimated the air volume displaced by the wings during 1 beating cycle to be $0.51 \pm 0.03\,cm^3$, which corresponds to a volume of $116.8 \pm 5.8\,cm^3\,s^{-1}$ at a wing beat frequency of 230 Hz. The authors then tested realistic scenarios of exposure for bees flying through a pesticide cloud at different concentrations. In the worst-case scenario, the dose accumulated during the flight reached $525\,ng\,bee^{-1}\,s^{-1}$. These results show that the procedure used to assess the risk posed by contact with pesticides could be improved by accounting for wing exposure. *Environ Toxicol Chem* 2015;34:1983–1988. © 2015 SETAC

Keywords: Neonicotinoids Pyrethroids Honey bee Wing exposure Risk assessment

INTRODUCTION

Pesticides are used in agriculture to protect crops from pests and should be assessed for their potential risk to the environment, particularly to nontarget organisms. Risk assessment procedures are based on comparing the levels of exposure to the substances and the toxicity of these substances, which can occur through different routes of exposure (contact, oral, and inhalation) [1,2]. Among nontarget organisms, honey bees are pollinators of high agro-environmental, economic, and scientific interest and are thus considered a relevant bioindicator species in the pesticide registration procedure [3]. During their foraging activity, honey bees visit plants in a radius as large as 12 km around the hive, which exposes them to a significant variety of pollutants, including pesticides, especially systemic insecticides [4,5]. Honey bees act as pollinators, thereby contributing to plant biodiversity and increasing the qualitative and quantitative yields of fruits and vegetables [6,7]. In the context of scientific research, the honey bee is now regarded as one of the most relevant models to study cognitive functions such as vision, orientation, memory, and brain plasticity [8,9].

Assessing the risk a pesticide poses to the honey bee through acute contact is based on the field application rate (g/ha) and the level of toxicity (μg/bee) [10] assessed through topical contact with the thorax [3,11]. Recently, it has been demonstrated that it is possible to assess the exposure level of a bee (mass of active substance/bee) subjected to a pesticide spray if the apparent surface area of a bee and the application rate are known [12]. Moreover, the toxicity induced by a spray applied to the bee's whole body is similar to that induced by a dose applied to the thorax, as derived from the field application rate and the exposure surface area of a bee.

The wings comprise a large proportion of the bee surface area (∼one-third of the total surface area) [12]. However, the possibility of systemic toxicity induced by contact with the wings as a route of exposure has never been considered. Wing exposure may be relevant because the circulatory system of the honey bee is well developed in the wings [13]. If such a systemic toxicity could occur, it would be necessary for pesticide risk assessment to consider an additional exposure scenario of a bee flying through a cloud of pesticide following a spray application. The dose received through the wings would depend on the pesticide concentration in the cloud, the transfer rate from the cloud to the bee, and the time the bee spends in the cloud.

In the present study, we aimed to determine whether systemic toxicity could occur after the bee's wings came into contact with a pesticide. As model substances, we selected 6 insecticides: 3 neonicotinoids (clothianidin, imidacloprid, and thiamethoxam) and 3 pyrethroids (deltamethrin, esfenvalerate, and lambda-cyhalothrin). Because absorption through the teguments depends significantly on the hydrophobicity of the substances, these insecticides were chosen for their contrasting physicochemical properties, with a relatively high water solubility for neonicotinoids and a low water solubility for pyrethroids [14]. Moreover, these insecticides are very toxic to bees, which enables us to demonstrate toxicity via wing exposure. In addition, neonicotinoids are systemic insecticides that are suspected to be involved in the decline of the honey bee [15–17].

In the present study, we first investigated the toxicity that could be induced by contact with the wings and compared it with the toxicity induced by contact with the thorax. Then, we considered the exposure of bees flying in a pesticide cloud based on realistic cases of exposure [18,19].

MATERIALS AND METHODS

Chemicals

Clothianidin (99% pure), deltamethrin (98% pure), esfenvalerate (99% pure), imidacloprid (99% pure), lambda-cyhalothrin

All Supplemental Data may be found in the online version of this article.

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(98.5% pure), and thiamethoxam (98.5% pure) were obtained from TechLab. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich.

Honey bee collection

Honey bee workers (*Apis mellifera*) were collected from the reserve frames of a hive body of healthy colonies ($\geq$30 000 individuals). Bees were monitored carefully for their sanitary state and placed in the experimental apiary of the French National Institute for Agricultural Research (Avignon) Bees and Environment research unit. After light anesthesia with CO_2, the bees were distributed into plastic cages (10.5 cm $\times$ 7.5 cm $\times$ 11.5 cm) in groups of 30 individuals and placed in the dark in a thermostat-controlled chamber at $28 \pm 2\,^{\circ}C$ and $60 \pm 10\%$ relative humidity [20]. The ambient temperature of the thermostat-controlled chamber was that preferred by the honey bees during the night [21,22]. The bees were fed ad libitum with candy (Apifonda plus powdered sugar) and water [23].

Exposure to pesticides

The acute toxicities of clothianidin, deltamethrin, esfenvalerate, imidacloprid, lambda-cyhalothrin, and thiamethoxam via contact with the thorax and wings were determined. The honey bees were divided randomly into 2 groups: 1 treated on the thorax and the other treated on the wings. For each substance, dose, and group, 8 replicates of 30 bees were performed systematically (Supplemental Data, Table S1). The active substances were diluted in DMSO, and the controls were treated with pure DMSO. The bees were treated according to the European and Mediterranean Plant Protection Organization procedure recommended by the European Commission [3,11]. Mortality was checked 24 h, 48 h, 72 h, 96 h, and 120 h after exposure. All motionless bees that did not respond to 3 consecutive contact stimulations were considered dead [24].

Basic kinematic model of a honey bee wing beat

During flight, the wings on the same body side can be considered as a single wing because they are linked with hooks that are present on the edge of the small wing [13]. The shape of the wings was determined using the ImageJ software package using pictures taken under a microscope (JENOPTIK ProgRes CF scan; Nikon MULTIZOOM AZ 100). The wings were divided into 28 segments perpendicular to the wing radius (0; R). The width of the wing was modeled and defined by 3 polynomial regressions as functions of the wing length (Figure 1)

$$f(x);\,(x \in \mathbb{R}|0 \leq x \leq a) \tag{1}$$

$$g(x);\,(x \in \mathbb{R}|a \leq x \leq b) \tag{2}$$

$$h(x);\,(x \in \mathbb{R}|b \leq x \leq R) \tag{3}$$

In the present study, the honey bee flight model was simplified slightly, because the goal of the present study was to approximate rather than precisely determine the volume of air the wings displaced. The simplifications were based on the insect flight descriptions of Ellington [25] and the more recent descriptions of Zhang et al. [26] and Altshuler et al. [27]. The wing deviation φ (Equation 4) with respect to the stroke plane was regarded as negligible, and the stroke angle $\phi = p(t)$ (Equation 5) and attack angle $\alpha = q(t)$ (Equation 6) were modeled with sinusoidal functions, based on the study of Altshuler et al. [27] (Figure 2)

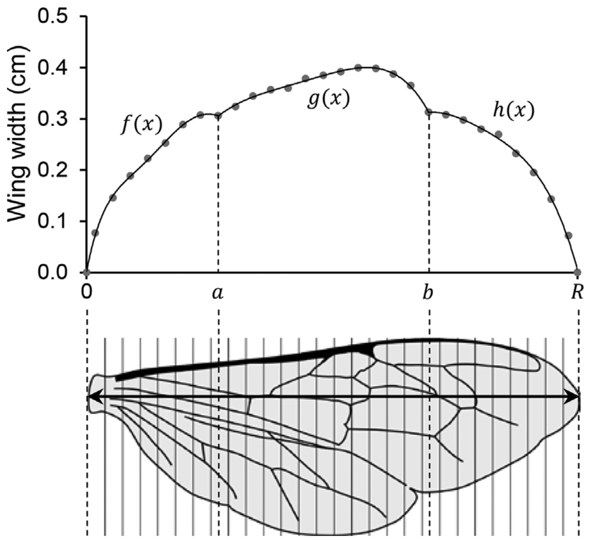

Figure 1. Modeling of the wing surface. For each wing, the shape of the wing was delineated from pictures taken under a microscope. The wing was divided into 28 segments (each with a width of one-third mm) perpendicular to the radius axis of the wing [0; R]. The mean width of each segment was measured, and the surface of the wing was modeled in 3 sections ([0; a], [a; b], [b; R]). The grey dots represent the actual data, whereas the black curve represents the modeled data. Example equations for the wings used in the modeling are: $f(x) = 1865.3x^5 - 1569.7x^4 + 475.58x^3 - 66.1x^2 + 5.2483x + 0.0016$; $r^2 = 0.9995$ $g(x) = -35.23x^4 + 57.52x^3 - 35.175x^2 + 9.8453x - 0.7211$; $r^2 = 0.994$ $h(x) = -95.73x^4 + 288.44x^3 - 327.47x^2 + 165.49x - 31.023$; $r^2 = 0.9976$

$$\varphi = 0 \tag{4}$$

$$\phi = \phi_0 \cos(2\pi t) \tag{5}$$

$$\alpha = \alpha_0 \left(\sin(2\pi t) + \frac{1}{8}\sin(6\pi t) \right) + K \tag{6}$$

where ϕ_0 is half of the total wing stroke amplitude (in this case 45.5°), α_0 is half of the total angle of attack amplitude (in this case 59.5°), K is the attack angle at the point of departure of the flapping period (in this case –3.5°), and t $(t \in \mathbb{R}|0 \leq t \leq 1)$ is the time fraction of the flapping period. To simplify the

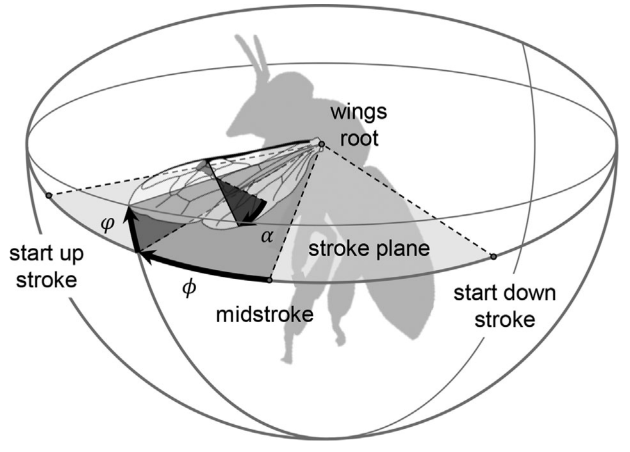

Figure 2. Description of the deviation (ϕ), stroke (φ), and attack (α) angles of the honey bee wings. Note that ϕ, φ, and α vary with the phase of the beating. The figure is inspired by the work of Sane and Dickinson [33] and Lentink and Dickinson [34].

calculation of the volume generated by a wing beat, the flapping period of the wing can be divided into m ($m \in \mathbb{N}$) time periods (in this case, 1000), where each period is defined between 2 times, $\frac{j}{m}$ and $\frac{j+1}{m}$, with $(j \in \mathbb{N} | 0 \leq j \leq m-1)$. The stroke angle $\Delta\phi_j$ (Equation 7) the wing travelled during each period can be defined as

$$\Delta\phi_j = |p\left(\frac{j+1}{m}\right) - p\left(\frac{j}{m}\right)| \tag{7}$$

The mean attack angle $\overline{\alpha_j}$ (Equation 8) of the wing during each period can be defined as

$$\overline{\alpha_j} = \frac{q\left(\frac{j}{m}\right) + q\left(\frac{j+1}{m}\right)}{2} \tag{8}$$

Combining Equations 1, 2, 3, 7, and 8, the volume $V_{\Delta\phi_j}$ (Equation 9) the wing generated in the $\Delta\phi_j$ space interval can be calculated using Shell's integration method corrected with the cosine of the mean attack angle $\overline{\alpha_j}$

$$V_{\Delta\phi_j} = \cos(\overline{\alpha_j})\frac{\Delta\phi_j}{360}2\pi\left(\int_0^a xf(x)dx + \int_a^b xg(x)dx + \int_b^R xh(x)dx\right) \tag{9}$$

Then, the volume V_w (Equation 10) generated by the 2 pairs of wings during a complete beating period can be expressed as follows

$$V_w = 2 \times \sum_{j=0}^{m-1}\cos(\overline{\alpha_j})\frac{\Delta\phi_j}{360}2\pi\left(\int_0^a xf(x)dx + \int_a^b xg(x)dx + \int_b^R xh(x)dx\right) \tag{10}$$

Data analysis

Statistical analyses were performed on the most stabilized dose–response relationship (120 h toxicity profiles) using the R software package (Ver 3.1.3). For each active substance and dose, the mortality difference between the thorax and wing exposures was determined using Fisher's exact tests (Supplemental Data, Dataset S1).

Median lethal dose calculation

Determining the median lethal dose (LD50) was based on the benchmark dose approach developed by the US Environmental Protection Agency and currently used in human risk assessment [28]. This approach consists of selecting the dose–response model (Gamma, Logistic, LogLogistic, LogProbit, Multistage, Probit, Quantal-Linear, and Weibull) that best fits the experimental data. For data that obey a binomial distribution, such as mortality, the models describe the probability that a death occurs as a function of the exposure level. The models were classified by their Akaike's Information Criterion (AIC) values, and the model with the lowest AIC was selected for determining the LD50 for each exposure modality (Supplemental Data, Table S2).

RESULTS

Thorax exposure versus wing exposure

The possibility that toxicity could be induced by a substance coming into contact with the wings was tested using 6 insecticides: 3 neonicotinoids (clothianidin, imidacloprid, and thiamethoxam) and 3 pyrethroids (deltamethrin, esfenvalerate, and lambda-cyhalothrin; Table 1). The mortality rates induced by the insecticides' contact with the wings and thorax were compared (Figure 3 and Table 1). Except for lambda-cyhalothrin (Figure 3F), the thorax and wing toxicity profiles were significantly different, with wing exposure inducing lower toxicity. The shapes of the thorax and wing dose–mortality relationships were globally similar, except for those of deltamethrin (Figure 3D). Although differences were observed between the toxicity profiles of the active substances, the LD50 obtained with the wing and thorax contacts were on the same order of magnitude for all substances and varied from 0.99 to 2.23 between the 2 exposure modes.

Volume displaced by the wings

Because the mortality rates elicited by thorax and wing exposures were similar, contact with the wings represents a possible route of exposure during spraying. Thus, to estimate the potential exposure through the wings during flight, the total volume (V_w) of air generated by the 2 pairs of wings during a complete beating period was determined (Equation 10 and Figure 4). Notable is that the total volume of air displaced could be divided into 2 volumes that corresponded to the down-stroke and up-stroke phases (Figure 4). The volume displaced by the 2 pairs of wings was thus estimated to be $0.51 \pm 0.03\ cm^3$ per beat ($n = 8$; Supplemental Data, Table S3), which represents a volume of $116.8 \pm 5.8\ cm^3\ s^{-1}$, assuming a wing beat frequency of 230 Hz [27,29]. It should be noted that the volume displaced per second is almost equivalent to the volume of a sphere with a radius of 3 cm.

Table 1. Toxicity of insecticides following exposure on the thorax or wings[a]

Active substance	Solubility in water (mg/L)	Log K_{OW}	LD50 (ng bee^{-1}) Wings	Thorax	Ratio W/T
Thiamethoxam	4.1×10^3	-1.30×10^{-1}	27.03	12.13	2.23
Imidacloprid	6.1×10^2	5.70×10^{-1}	26.55	25.10	1.06
Clothianidin	3.4×10^2	9.05×10^{-1}	36.49	25.84	1.41
Lambda-cyhalothrin	5.0×10^{-3}	5.50	40.46	40.98	0.99
Esfenvalerate	1.0×10^{-3}	6.24	192.28	175.46	1.10
Deltamethrin	2.0×10^{-4}	4.60	92.11	44.40	2.07

[a]The water solubility and the octanol−water partition coefficient (log K_{OW}) of the pesticides were obtained from the International Union of Pure and Applied Chemistry Pesticides Properties DataBase (IUPAC PPDB) [14]. The ratio W/T corresponds to wing LD50 / thorax LD50. The median lethal dose (LD50) was determined at 120 h for all active substances.

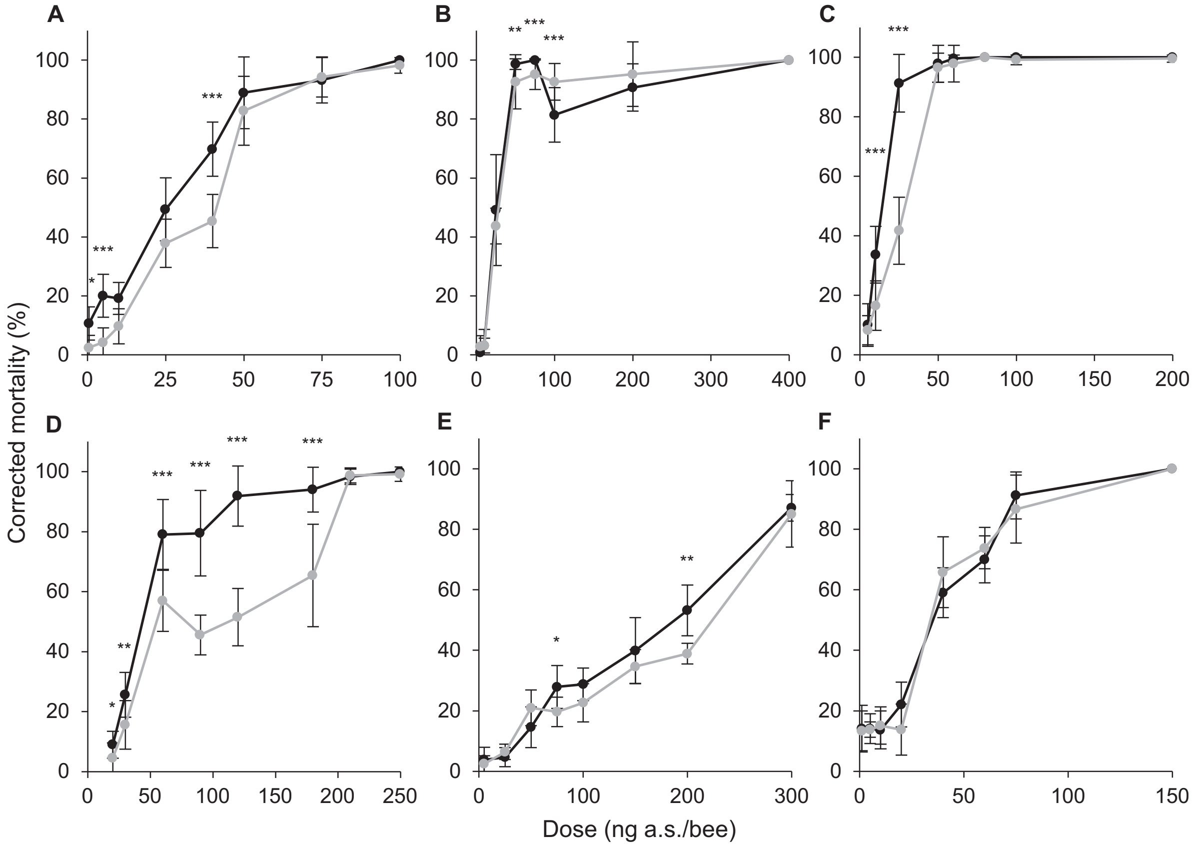

Figure 3. Toxicity profiles of insecticides in bees exposed by contact on the thorax and wings. Bees were exposed to different doses of active substances (a.s.) by topical applications of 1 μL of the insecticide solutions on the thorax or wings: (**A**) clothianidin, (**B**) imidacloprid, (**C**) thiamethoxam, (**D**) deltamethrin, (**E**) esfenvalerate, and (**F**) lambda-cyhalothrin. The black and grey dots correspond to the thorax and the wings, respectively; the error bars indicate the standard deviations. The mortality of the controls did not exceed 6.25% at 120 h for any of the active substances tested. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Scenarios of exposure to pesticides for a flying honey bee

Different scenarios of exposure were investigated by modulating the transfer rate and the actual pesticide concentrations in the spray clouds reported in the literature (Table 2 and Figure 5). The different scenarios showed that flight could drastically change the exposure level. In the worst-case exposure scenario, at transfer rates of 0.1, 0.5, and 1, a bee flying in a pesticide cloud is subjected through the wings to exposure rates of 52.5 ng $bee^{-1}\,s^{-1}$, 262.5 ng $bee^{-1}\,s^{-1}$, and 525 ng $bee^{-1}\,s^{-1}$, respectively.

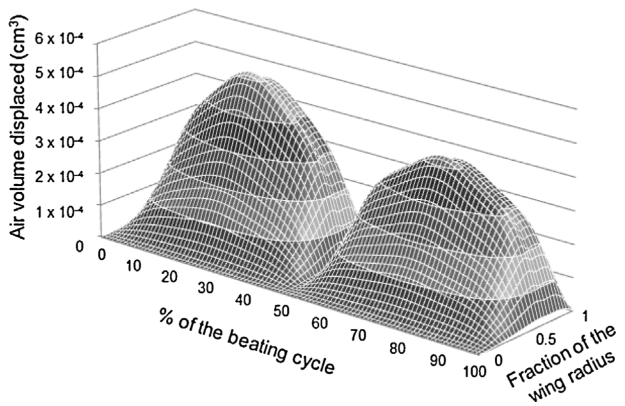

Figure 4. Air volume displaced by honey bee wings during 1 wing beating cycle. An example of the modeling for the beating of wings from 1 of the bees studied is presented in Figure 4.

DISCUSSION

The present study is the first to demonstrate pesticide toxicity by wing contact in insects, especially in the honey bee. Pesticides applied to honey bee wings elicit toxicities similar to those observed after contact with the thorax. This systemic toxicity induced by wing contact could be explained in part by the extensive vascularization of honey bee wings and the close cuticle density of wings and teguments [13,30]. The close toxicity profiles obtained with the 2 exposure routes suggest that the substances might be distributed in the bee body by wing contact as rapidly as by thorax contact and the toxicokinetics of the substances might not differ strongly between the 2 exposure routes. In addition, the distance between the exposure site and the target tissue of pesticides appears to be critical in the shock action of the pesticides [31]. Thus, because the wings are anchored on the thorax, it is not surprising that the toxicities induced by wing and thorax contacts are very similar. The toxicity induced by contact with the wings appears to be independent of the hydrophobicity of the active substance; this result might be explained by the relatively neutral properties of honey bee wings, which exhibit a contact angle with water droplets of 92° [32].

The wings comprise a large fraction of a bee's body surface area (one-third of the total surface area; $1.06 \pm 0.03\,cm^2$) [12]. Contact with the wings, therefore, represents a very significant route of exposure to pesticides. Hence, a bee flying through a pesticide cloud might be exposed to higher levels of pesticides than a motionless bee. This exposure scenario, although not focused on wing exposure, was considered by the European

Table 2. Dose received by the wings of a bee flying in a pesticide spray cloud[a]

$[\text{Pesticide}]_{air}$ ($ng\ cm^{-3}$)	Reference	Transfer rate 0.005	0.01	0.05	0.1	0.5	0.75	1
19×10^{-5}	[35]	1.1×10^{-4}	2.2×10^{-4}	1.1×10^{-3}	2.2×10^{-3}	1.1×10^{-2}	0.02	0.02
10×10^{-4}	[36]	5.8×10^{-4}	1.2×10^{-3}	5.8×10^{-3}	1.2×10^{-2}	0.06	0.09	0.12
8.6×10^{-3}	[37]	5.0×10^{-3}	1.0×10^{-2}	0.05	0.10	0.50	0.75	1.0
6×10^{-2}	[38]	0.03	0.07	0.34	0.67	3.4	5.0	6.7
4.5×10^{-1}	[39]	0.26	0.53	2.6	5.3	26.5	39.7	53.0
4.5	[40]	2.6	5.3	26.3	52.5	262.7	394.1	525.4

[a]The doses received by the wings of bees ($ng\ bee^{-1}\ s^{-1}$) were estimated based on the measured pesticide concentrations reported in the literature and the different scenarios of transfer rates. Note that the pesticide concentrations varied by approximately 1 order of magnitude each time.

Food and Safety Authority in 2012 and 2013 [18,19]. It was not retained in the risk assessment procedure, however, because information regarding its occurrence was lacking.

The present study highlights the need to gather information regarding the occurrence of such exposure cases. Considering a wing beat frequency of 230 Hz and a stroke amplitude of 91° [27], the estimated volume of air displaced by the wings is $116.8 \pm 5.8\ cm^3\ s^{-1}$. This estimate appears to be reasonable, because a wing beat frequency of 250 Hz and a total stroke amplitude of 120° have been reported [26]. Thus, the exposure for a motionless bee that takes off through a pesticide cloud immediately after being subjected to pesticide spraying can be estimated as follows

$$\begin{aligned} TD &= ID + BD + WD \\ &= ID + BD + (V_w \times f \times t \times C \times T_w) \end{aligned} \qquad (11)$$

where *TD* is the total dose the bee received expressed in nanograms per bee; *ID* is the individual dose received by a motionless bee during spraying expressed in nanograms per bee [12]; *BD* is the dose received by the body during the flight, which depends on the flight speed, the attack angle of the body, the concentration of the pesticide cloud, the time spent in the pesticide cloud, and the transfer coefficient of the pesticide; *WD* is the dose received by the wings during the flight; V_w is the volume displaced by the wings expressed in centimeters cubed per beat (in the present study, $0.51 \pm 0.03\ cm^3$ per beat); f is the wing beat frequency (in the present study 230 Hz); t is the time spent in the pesticide cloud by the bee expressed in seconds; C is the pesticide concentration in the cloud expressed in nanograms per centimeter cubed; and T_w is the transfer coefficient of the pesticide from the cloud to wings.

The value of T_w varies among pesticides and is ≤ 1, but it can be fixed to 1 to consider the worst-case scenario. Notably, the potential effects depend on the toxicity of the active substance at given exposure levels. However, the close toxicity profiles obtained by wing and thorax exposures with insecticides of contrasting physicochemical properties strongly suggest that the main factor responsible for the toxicity induced by wing contact is the intrinsic toxicity of a pesticide, not its physicochemical properties. Hence, it is possible to expect similar classifications of substances according to their toxicity induced by thorax or wing contact. In this case, neonicotinoids, phenylpyrazoles, and pyrethroids might still be the most toxic substances to the honey bee [17]. Thus, considering that exposure during flight is particularly important, it is necessary to refine the overall assessment procedure for bees subjected to pesticide exposure by spraying. Beyond the honey bee, if the toxicity of a pesticide to other insects depends on the vascularization and the beat frequency of the wings, the assessment of exposure to pesticides should be reconsidered for nontarget insects.

The present study stresses the importance of considering the wings as a relevant route of exposure to pesticides. Further studies should focus on complementary aspects that could help improve the assessment of the risk of pesticides to bees. Particularly important information needed includes the probability of bee flights occurring in pesticide clouds, time spent in clouds in different exposure scenarios, and transfer rates of pesticides through wings.

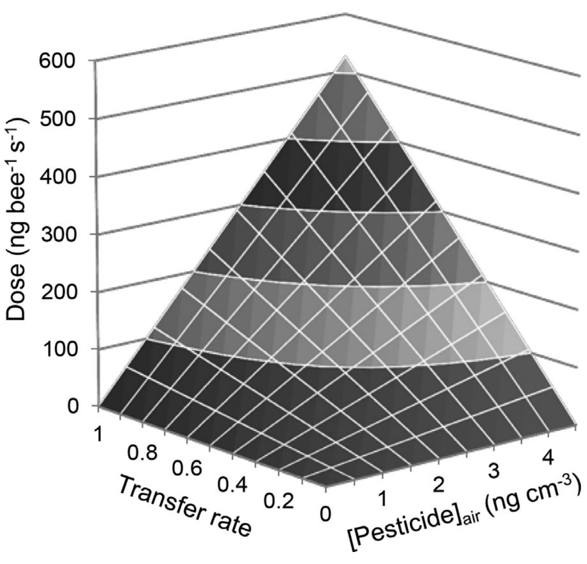

Figure 5. Level of exposure to a pesticide during wing beating. Different scenarios of exposure were considered based on realistic concentrations of pesticides in the air and rates of transfer from the pesticide spray cloud to the bees' wings.

SUPPLEMENTAL DATA

Tables S1–S3. (45 KB DOCX).
Dataset S1. (19 KB XLSX).

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Data availability—Data from Figure 3 are available in figshare at the following DOI: http://dx.doi.org/10.6084/m9.figshare.1296229. The data from the other figures are provided in the manuscript or the supplemental data.

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