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**Original article**

# *Tilia* trees: toxic or valuable resources for pollinators?

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**Abstract –** To investigate whether *Tilia* trees are poisonous or valuable resources for bee visitors, we determined the nectar and pollen quantities and composition of the four main *Tilia* species planted in Western Europe (*T. cordata*, *T. platyphyllos*, *T. tomentosa*, and *T.* × *europaea*). We developed a new method to detect nicotine in nectars. We observed insect visitor diversity and abundance. We also assessed bumblebee death when individuals were only fed with *Tilia* flowers. No traces of mannose or nicotine, incriminated in the *Tilia* toxicity, have been detected in the nectars of the studied species. Huge numbers of insect visitors, mainly bees and syrphids, visited the trees which offer large numbers of flowers, plenty of sugar rich nectar, and protein-rich pollen. Bumblebees only fed with *Tilia* flowers did not present any particular mortality. We discuss the different hypotheses of the supposed toxicity and propose future research to solve this debate.

**linden trees / *Tilia tomentosa* / nicotine / mannose / bees / nectar / pollen**

## 1. INTRODUCTION

Pollinator decline is a worldwide phenomenon and is commonly attributed to anthropogenic causes such as habitat destruction and fragmentation (including urbanization), floral resource shortages, application of pesticides, introduction of parasites, or biological invasions (Goulson et al. 2015). Urbanization represents a major proposed cause of insect decline, particularly through alteration of food resources and nesting sites (McKinney 2008; Bates et al. 2011).

However, in cities, mass flowering trees that represent huge floral resources during several weeks may constitute valuable food for generalist insects (Hunter and Hunter 2008; Somme et al. 2016).

In this context, different species of linden or lime trees (*Tilia* spp., Malvaceae) are often planted as valued park trees along avenues and roads as they offer good compromises in urban plantings (Pawlikowski 2010; Weryszko-Chmielewska and Sadowka 2010). *Tilia* spp., with about 30 species, are deciduous trees from the temperate zones of the Northern Hemisphere (Pigott 2012; Ivanov et al. 2014). Besides the common names given to *Tilia* species, another widely used name in Europe is "bee tree" and *Tilia* trees are considered of high apicultural value (Anderson 1976). Bees collect predominantly pollen (Free 1970; Illies 2016) even if they also visit flowers for nectar (Weryszko-Chmielewska and Sadowka 2010; Pawlikowski 2010; Gašić et al. 2014; Illies 2016).

Two native species are present in northwestern Europe, *Tilia cordata* Mill., the small-leaved lime, and *Tilia platyphyllos* Scop., the

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large-leaved lime (Tutin et al. 1968; Radoglou et al. 2009). They form a natural inter-specific hybrid, *T.* × *europaea* L., the common lime. *Tilia tomentosa* Moench, the silver lime, grows only in southeastern Europe (Tutin et al. 1968). All species flower profusely in June and July; their blooming periods overlap to a large extent (Anderson 1976). *Tilia platyphyllos* is the first, and *T. tomentosa* is the last species to flower. This flowering at different times can provide forage for insects over a period of 6 weeks (Weryszko-Chmielewska and Sadowka 2010). The numerous fragrant pale yellow-green open flowers are organized in cymes of 4 to 15 flowers (Tutin et al. 1968; Anderson 1976). They have numerous anthers and offer easily accessible nectar to generalist insects with short tongues, especially flies, syrphids, and bees (Anderson 1976; Pigott 1991). Bees are the main visitors and pollinators, even if other generalist insects are also abundantly observed on *Tilia* trees (Knuth 1908; Anderson 1976; Pigott 1991). Due to their numbers, their morphology, and their behavior, they contact reproductive organs and insure pollination combining pollen removal from anthers, transport, and deposition on stigmas (Anderson 1976). *Tilia* species require insect pollination, spontaneous self-pollination being unlikely due to herkogamy and protandry of flowers (Anderson 1976; Illies 2016).

For several years, the toxicity of *Tilia* tree species for insect visitors is under debate as dead bees have been observed under the trees in different countries (Geissler and Steche 1962; Madel 1977; Mühlen et al. 1992, 1994; Baal et al. 1994; Surholt and Baal 1995; Illies and Mühlen 2007; Pawlikowski 2010; Illies 2016; Koch and Stevenson 2017). More frequent deaths were observed among bumblebees than among honeybees (Mühlen et al. 1994; Pawlikowski 2010; Illies 2016; Argoti 2016).

Two main hypotheses have been proposed to explain how *Tilia* trees may cause bees to die. The first hypothesis posits that flowers of *Tilia* are poisonous (Crane 1977; Argoti 2016; VKM 2017). Nectar is the principal source of carbohydrates for most flower visiting insects (Baker 1977; Nicolson 2011). Certain sugars, which disturb carbohydrate metabolism in bees (Arnold et al. 1974), have been incriminated in the toxicity of *Tilia* trees and specifically mannose (Crane 1977; Madel 1977; Argoti 2016, but see Baal et al. 1994 and Krasenbrink et al. 1994). Mannose has been detected in nectar of other plant species such as *Tordylium apulum* (Apiaceae) or *Cistus salvifolius* (Cistaceae) (Petanidou 2005). Nectar can also contain low concentrations of other potentially toxic compounds, such as alkaloids, phenolics, or other compounds (Tiedeken et al. 2014). Particularly, alkaloids are highly toxic to bees across a wide range of concentrations (Singaravelan et al. 2006). One alkaloid, nicotine, has been incriminated to explain the toxicity of *Tilia* species. Nicotine is toxic for adult honeybee workers (Baracchi et al. 2015) at LD50 concentration of 2000 ppm (12.3 mmol/L) (Detzel and Wink 1993) while a concentration of 50 ppm already affects larval survival (Singaravelan et al. 2006). Regarding bumblebees, Tiedeken et al. (2014) reported that 16.2 ppm (0.1 mmol/L) of nicotine is deterrent for *Bombus terrestris* even if no change in the survival of individuals has been detected. Nicotine has been detected, and its concentration varied from 0.1 to 3.0 ng/μL in nectar of two genera *Nicotiana* and *Tilia* species (Kessler et al. 2010; Tadmor-Melamed et al. 2004; Baracchi et al. 2015).

Besides toxicity of floral resources, a second hypothesis claims that *Tilia* flowers do not contain sufficient nectar to feed insect visitors. In this case, visitors starve to death (Mühlen et al. 1994; Baal et al. 1994; Surholt and Baal 1995; Illies 2016; VKM 2017; Koch and Stevenson 2017). Nectar content in *Tilia* flowers is effectively highly variable in time with a decrease in nectar volume throughout the day and towards the end of the season (Illies 2016).

Our objective was to detect whether *Tilia* species are toxic for their main bee pollinators and to compare the concentration of the incriminated toxic compounds, mannose and nicotine, among the principal planted *Tilia* species in Western Europe, *T. cordata*, *T. platyphyllos*, their hybrid *T.* × *europaea*, and the most controversial introduced *T. tomentosa*.

Here we analyzed nectar and pollen composition, including the presence of mannose and

nicotine in nectar. We developed a new method to detect nicotine in nectars. We also observed the behavior and survival of bees foraging on *Tilia* trees.

We addressed the following questions: (i) Do the different *Tilia* species offer nectar and pollen in similar quantities and of similar composition? (ii) Do the nectars of *Tilia* species contain mannose, other minor toxic sugars and/or nicotine? (iii) Do the different *Tilia* species attract the same insect visitor species and with similar relative abundances? (iv) Do bumblebees that are exclusively fed on *Tilia* nectar present an increased mortality?

## 2. MATERIALS AND METHODS

### 2.1. Plant material

We sampled four *Tilia* species planted in Louvain-la-Neuve, Belgium: *Tilia cordata*, *T. platyphyllos*, their hybrid *T.* × *europaea*, and *T. tomentosa*. *Tilia* trees were sampled in 2016 and 2017 along Avenue Baudouin 1er and in different parks at the University Campus (50° 39′ 58″ N; 4° 37′ 9″ E).

Tree height, trunk diameter, crown volume, and the number of flowers per tree were estimated on three trees per species. The number of flowers per tree was estimated by counting the open flowers on three flowering parts of the crow (known volume) per tree and was expressed as the number of flowers per cubic meter of crown.

A total of 15 flower buds per species were dissected (5 per tree from 3 trees) to assess the number of anthers and of pollen grains per flower. Pollen grains were counted for one stamen per flower. The anthers were individually crushed in a microcentrifuge tube, containing 200 μL of Alexander's stain. They were then mixed and sonicated to disperse the pollen grains in the solution. The number of pollen grains was counted in three subsamples of 4 μL per anther under light microscopy (Eclipse E400; Nikon, Amsterdam, The Netherlands). Numbers of pollen grains per flower were obtained by multiplying the numbers of pollen grains per anther by the numbers of anthers of a flower.

### 2.2. Pollen composition

#### *2.2.1. Pollen sampling*

At the peak of flowering (i.e., late June to mid-July), branches with unopened flower buds were harvested from five trees per species. Branches, placed in tap water, were kept for one night at room temperature (approx. 20 °C) in the lab. On the next morning, stamens were extracted from newly opened flowers. Stamens were stored at − 20 °C and subsequently dried at room temperature for 12 h before pollen was removed using a sieve (Sieve 3″, Brass-Stainless, Full Height, 80 μm). Pure pollen samples were pooled to obtain 200 mg samples sufficient for analyses and stored at − 20 °C until use.

#### *2.2.2. Chemical analyses*

Chemical analyses were performed in triplicate, as in other recent studies (Weiner et al. 2010; Vanderplanck et al. 2011, 2014; Somme et al. 2015; Villette et al. 2015).

The polypeptide content (molecular weight > 10,000 Da) was quantified from 5 mg dry pollen of each species (Vanderplanck et al. 2014). The polypeptide purification protocol combined washes and a phenol/sodium dodecylsulfate extraction. The quantification of total polypeptide content was performed using the bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Thermo Scientific) according to manufacturer instructions and using bovine serum albumin (BSA) for standard curve.

The amino acid concentrations were quantified from 3 mg dry pollen of each species (Vanderplanck et al. 2014). Total and free amino acids were extracted separately and measured by an ion-exchange chromatography (Biochrom 20 plus amino-acid analyzer). For both extractions, norleucine was used as the internal standard.

The phytosterol content was quantified from 15 mg dry pollen of each species (Vanderplanck et al. 2011). After extraction and derivatization of the sterols into their respective trimethylsilyl ethers, these were separated by gas–liquid chromatography. The total phytosterol content was determined by considering all quantifiable peaks

of sterols that eluted between cholesterol and betulin (internal standard). Identifications were achieved by comparing the relative retention times (β-sitosterol–TMS = 1.00) with those of sunflower oil reference.

### 2.3. Chemical composition of nectar

#### *2.3.1. Nectar sampling*

Branches with unopened flower buds were harvested from five trees per species at the same time that pollen was collected. Nectar collection from flowers on the branches was conducted in the lab (Somme et al. 2016). Branches, placed in tap water, were kept for one night at room temperature (approx. 20 °C). On the next morning, nectar was extracted from newly opened flowers. This method avoids differences due to climatic conditions (temperature and relative humidity) in the field and previous visits to flowers by insects. For each tree species, nectar was collected from at least 50 (depending on the nectar volume) newly opened flowers with glass capillary tubes of 5 μL (Hirschmann®Laborgerate, Eberstadt, Germany). The nectar volume was estimated by measuring the length of the nectar column in the capillary tube. Nectar samples were stored at − 80 °C until chemical analyses.

#### *2.3.2. Sugar composition analyses*

The main sugar (sucrose, fructose, and glucose) and minor sugar (galactose, mannose, myo-inositol, raffinose, and kestose) concentrations were determined for each species in triplicate, from respectively 3 and 10 μL of nectar. After oxymation, sugar concentrations were determined by gas chromatography, with a PerkinElmer Autosystem XL equipped with a split injector (1/20; injection volume of 2 μL), a detector FID using a column Chrompack WCOT CP-SIK5 (25 m × 0.32 mm ID), and helium as the carrier gas (flow of 1 mL/min). The injector and detector temperatures were maintained at 270 and 350 °C, respectively. The total sugar content of nectar per flower (mg) was calculated by multiplying the volume of nectar (μL) by the total sugar concentration (mg/μL).

#### *2.3.3. Nectar nicotine analyses*

We developed a new method for nicotine detection in nectars (see Supplemental data for details). Nicotine purifications from nectar were performed by solid-phase extraction (SPE) on the weak cation exchanger cartridge Strata X-CW 33 μm (30 mg/3 mL) from Phenomenex. Nicotine was then quantified by HPLC-MS/MS (Abdallah et al. 2016). The protocols followed the recommendations of EMEA ( 2011).

We prepared dilutions of *Tilia* nectars on the basis of a potential nicotine concentration of 10 μM (1620 ng/mL), concentration detected by bumblebees (Tiedeken et al. 2014). We diluted the nectars in order to obtain a potential final concentration of 6.48 ng/mL, concentration in our calibration range. Internal standard (D4-nicotin) was added at a concentration of 200 ng/mL before SPE, its final concentration reaching 20 ng/mL in the injected solution**.** The deposited sample volume on the SPE cartridges was 500 μL.

### 2.4. Insect visitor behavior

#### *2.4.1. Insect visitor observations on the trees*

In 2016, insect visitors were observed on planted *Tilia* trees at Louvain-la-Neuve during observation periods of 15 min (a total of 70 periods), during several sunny days per species (beginning with *T. platyphyllos* in mid-June and ending with *T. tomentosa* in mid-July). The numbers of open flowers were assessed before any observation. All visitors were recorded and considered as pollinators when contacting the reproductive organs of a flower. As bumblebee species are not distinguishable in the field due to their similar morphology and color, we identified them in the field to morphotypes. Subsamples of bumblebee (Hymenoptera) and syrphid (Diptera) individuals were collected for precise identification in the lab.

One white sheet of 10 $m^2$ was deposited during 2 days and 10 h per day under one tree per species in full bloom to collect dead insects. After collection, insects were stored at − 20 °C until identification to species level in the lab.

#### 2.4.2. Bumblebee behavior and survival under controlled conditions

In 2017, we tested the survival of bumblebees fed only with flowers of *Tilia* in three independent experiments. Bunches of flowered branches of *T. cordata* and *T. tomentosa* were harvested on a minimum of five trees per species and placed in tap water under three individual mosquito nets (190 × 125 × 125 cm, Outillage de Saint Etienne, France) per *Tilia* species. Ten naïve worker bumblebees were placed under each net with the flowered branches for 1 week. For the three experiments, each set of bumblebees was sampled from a different hive of *Bombus terrestris* (three hives, Biobest, Westerloo, Belgium). Branches were replaced three times a week. We observed the survival of the bumblebees and their behavior twice a day (10:00 am and 04:00 pm). A control was conducted under the same conditions, with unflowered branches of *Tilia*. To compensate the absence of flowers in control conditions, "mix flower" pollen (François Dequit, Meris, France) and nectar substitute (Biogluc solution, Biobest, Westerloo, Belgium) were provided ad libitum.

### 2.5. Data analyses

Prior to analyses of variance (ANOVA), homoscedasticity and normality were checked using Bartlett and Shapiro-Wilk tests, respectively. One-way and two-way analyses of variance (ANOVA) were performed with SAS Enterprise Guide 7.1. Post-hoc analyses were performed using Tukey's tests.

Data are presented as means ± standard deviations.

## 3. RESULTS

### 3.1. Quantity of floral resources

The number of flowers per tree differed with *T.* × *europaea* and *T. tomentosa* producing more flowers per cubic meter (28,200 ± 17,100 and 30,100 ± 7000, respectively) than *T. cordata* (10,200 ± 7800) and *T. platyphyllos* (10,000 ± 4700; $F(3,32) = 10.33$; $p < 0.0001$).

*Tilia tomentosa* produced also a higher quantity of floral resources in comparison with the three other species (Figure 1a, b). The numbers of pollen grains per flower ranged from 2716 ± 450 × $10^3$ for *T. tomentosa* to 1647 ± 244 × $10^3$ for *T. cordata* ($F(3,32) = 13.42$; $p$ value < 0.0001; Figure 1a). The volumes of nectar per flower of *T. tomentosa* and *T. cordata* were twice higher than those of *T. platyphyllos* and *T.* × *europaea* ($F(3,56) = 125.93$; $p$ value < 0.0001; Figure 1b).

### 3.2. Chemical composition of floral resources

#### 3.2.1. Pollen composition

The total content of sterols in the pollen did not differ significantly ($F(3,6) = 0.12$; $p$ value = 0.9426) and averaged 4.5 ± 0.4 μg/mg (Figure 1c). However, the composition differed greatly among species (Table I). Pollen of *T. platyphyllos* was characterized by a high concentration of D7-avenasterol and a low concentration of 24 methylencholesterol in comparison with other studied species (Table I).

*Tilia platyphyllos* pollen had the highest concentrations in both total (322.1 ± 6.8 μg/mg, $F(3,8) = 82.84$; $p$ value < 0.0001) and essential amino acids (136.4 ± 3.6 μg/mg, $F(3,8) = 36.13$; $p$ value < 0.0001) whereas *T. tomentosa* pollen presented the lowest values (209.3 ± 5.6 μg/mg and 100.3 ± 2.8 μg/mg, respectively; Figure 1d). The concentrations in the different amino acids differed among species with *T. platyphyllos* showing the highest concentrations in all amino acids at the exception of cysteine, methionine and phenylalanine (Table I). The concentrations of polypeptides ranged from 33.4 ± 1.9 μg/mg in *T.* × *europaea* to 21.0 ± 2.8 μg/mg in *T. cordata* ($F(3,14) = 5.14$; $p$ value = 0.0133; Figure 1e).

#### 3.2.2. Nectar composition

*Sugar concentrationsTilia tomentosa* nectar sugar concentration was twice higher than *Tilia platyphyllos* and *T.* × *europaea* nectar and four times higher than *T. cordata* (Figure 1f). This difference was mainly due to sucrose concentration

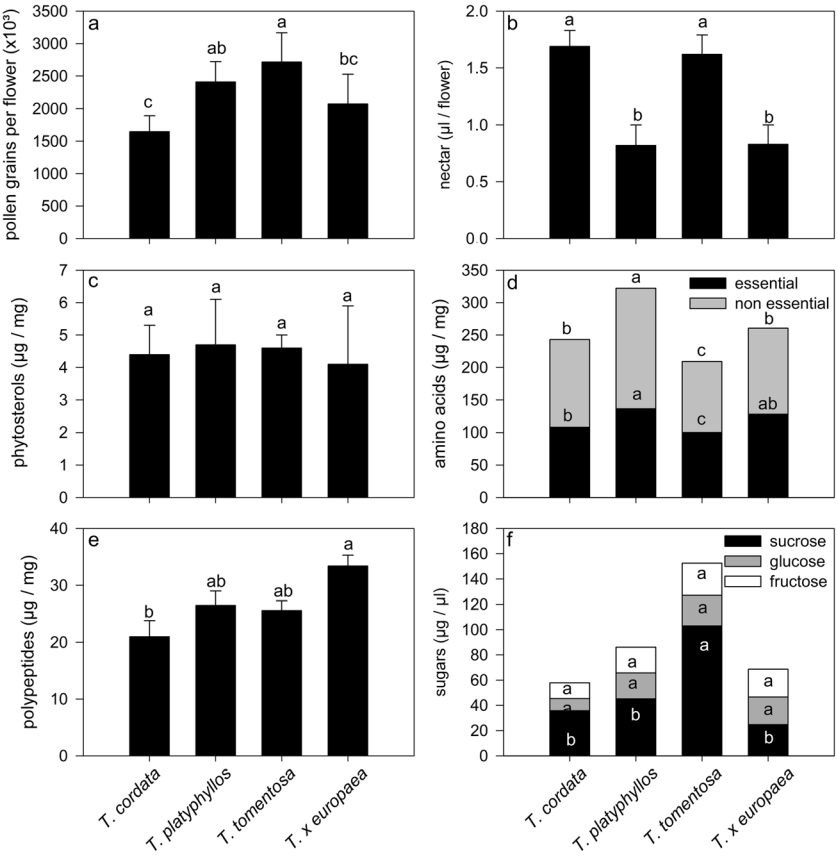


**Figure 1.** Pollen and nectar resources of the four studied *Tilia* species: pollen grains per flower ($\times 10^3$, **a**); nectar volume (µL/flower, **b**); phytosterol (**c**), amino acid (**d**), and polypeptide (**e**) concentrations (µg/mg) in pollen; sugar concentration in nectar (µg/µL, **f**). Different letters indicate significant differences among species.

($F(3,6) = 7.04$; $p$ value $= 0.0161$). The ratios sucrose/hexoses were significantly different among *Tilia* species, ranging from sucrose dominant nectar in *T. tomentosa* to hexose dominant nectar in *T.* × *europaea* (Figure 1f).

No trace of mannose (< 1.44 ng/µL) was detected whereas traces of galactose, myoinositol, and raffinose were detected in some of our studied *Tilia* species (Table II).

*Nicotine* We successfully developed a new method to detect nicotine in nectar, a high sugar rich solution, by SPE-HPLC-MS/MS (see Supplemental data). The extraction recovery was considered as very good with a mean of 95.32%, which was taken into account in all further concentration calculations (Table III).

The subsequent analyses of the collected *Tilia* nectars did not allow us to detect any trace of nicotine at LOD level (0.2 ng/mL) for the four species (Figure 2).

### 3.3. Insect visitor behavior and survival

#### *3.3.1. Abundance and diversity of visitors*

Diverse visitors, including honeybees, bumblebees, and syrphids, were observed on the four *Tilia* species. Honeybees were the main insect visitors except for *T.* × *europaea* ($\chi^2 = 165.69$; ddl $= 6$; $p$ value $< 0.0001$; Table IV).

**Table I.** Phytosterols (μg/mg) and amino acids (μg/mg) in pollen of the four studied *Tilia* species ($N = 3$ per species and analysis).

| Compounds | *T. cordata* | *T. platyphyllos* | *T. tomentosa* | *T.* × *europaea* | ANOVA |
|---|---|---|---|---|---|
| | | | Phytosterols | | |
| Cholesterol | 0.26 ± 0.15a | 0.18 ± 0.06ab | 0.02 ± 00.1b | 0.12 ± 0.06ab | $F(3,8) = 3.97$ $p$ value = 0.0528 |
| Cholestenone | 0.05 ± 0.02 | 0.01 ± 0.01 | 0.09 ± 0.08 | 0.01 ± 0.01 | $F(3,8) = 3.44$ $p$ value = 0.0719 |
| Desmosterol | 0.02 ± 0.02 | 0.06 ± 0.0.5 | 0.04 ± 0.01 | 0.03 ± 0.0 | $F(3,8) = 2.01$, $p$ value = 0.1915 |
| 24-methylenecholesterol + campesterol | 0.24 ± 0.08a | 0.01 ± 0.01b | 0.23 ± 0.02a | 0.33 ± 0.10a | $F(3,8) = 12.97$ $p$ value = 0.0019 |
| Stigmasterol | 0.13 ± 0.02 | 0.15 ± 0.02 | 0.13 ± 0.01 | 0.12 ± 0.03 | $F(3,8) = 1.27$ $p$ value = 0.3482 |
| D5-avesterol | 1.16 ± 0.21 | 0.76 ± 0.13 | 0.93 ± 0.05 | 0.87 ± 0.29 | $F(3,8) = 2.34$ $p$ value = 0.1499 |
| D7-stigmasterol | 0.06 ± 0.07 | 0.09 ± 0.09 | 0.01 ± 0.01 | 0.01 ± 0.01 | $F(3,8) = 1.38$ $p$ value = 0.3182 |
| D7-avenasterol | 0.10 ± 0.06 b | 1.44 ± 039 a | 0.11 ± 0.02 b | 0.56 ± 0.17 b | $F(3,8) = 25.35$ $p$ value = 0.0002 |
| B-sitosterol | 2.37 ± 0.50 | 2.00 ± 0.22 | 3.04 ± 0.27 | 2.06 ± 0.62 | $F(3,8) = 3.56$ $p$ value = 0.0669 |
| Amino acids | | | | | |
| Alanine | 11.66 ± 0.65c | 18.60 ± 0.50a | 10.75 ± 0.30c | 13.25 ± 0.42b | $F(3,8) = 157.80$ |
| Arginine | 13.43 ± 0.93b | 15.64 ± 0.36a | 10.91 ± 0.31c | 13.81 ± 0.51b | $F(3,8) = 33.56$ $p$ value < 0.0001 |
| Asparagine | 26.60 ± 1.17bc | 43.97 ± 1.02a | 25.34 ± 055c | 28.34 ± 0.83b | $F(3,8) = 266.41$ $p$ value < 0.0001 |
| Cysteine | 2.47 ± 0.63a | 0.02 ± 0.04c | 1.56 ± 0.06b | 2.18 ± 0.10ab | $F(3,8) = 34.73$ $p$ value < 0.0001 |
| Glutamine | 28.53 ± 1.49b | 43.57 ± 1.18a | 24.48 ± 0.60c | 29.60 ± 0.98b | $F(3,8) = 168.36$ $p$ value < 0.0001 |
| Glycine | 10.71 ± 0.56c | 16.63 ± 0.51a | 9.99 ± 0.26c | 12.52 ± 0.39b | $F(3,8) = 132.37$ $p$ value < 0.0001 |
| Histidine | 9.13 ± 0.48b | 10.77 ± 0.33a | 8.24 ± 0.25b | 11.05 ± 0.37a | $F(3,8) = 40.29$ $p$ value < 0.0001 |
| Isoleucine | 12.63 ± 0.68c | 17.20 ± 0.49a | 10.84 ± 0.33d | 15.59 ± 0.51b | $F(3,8) = 91.21$ $p$ value < 0.0001 |
| Leucine | 18.72 ± 0.96b | 25.24 ± 0.79a | 15.67 ± 0.43c | 19.65 ± 0.62b | $F(3,8) = 90.60$ $p$ value < 0.0001 |
| Lysine | 18.29 ± 0.91b | 24.05 ± 0.36a | 15.68 ± 0.39c | 18.83 ± 0.59b | $F(3,8) = 101.06$ $p$ value < 0.0001 |
| Methionine | 6.83 ± 0.38a | 0.66 ± 0.31c | 5.61 ± 0.20b | 7.37 ± 0.35a | $F(3,8) = 275.65$ $p$ value < 0.0001 |
| Phenylalanine | 13.29 ± 0.73a | 9.42 ± 0.03c | 10.76 ± 0.35b | 14.27 ± 0.51a | $F(3,8) = 65.48$ $p$ value < 0.0001 |
| Proline | 22.12 ± 1.05b | 24.59 ± 0.65a | 16.63 ± 0.75c | 21.68 ± 0.61b | $F(3,8) = 53.97$ $p$ value < 0.0001 |

**Table I** (continued)

| Compounds | *T. cordata* | *T. platyphyllos* | *T. tomentosa* | *T. × europaea* | ANOVA |
|---|---|---|---|---|---|
| Serine | 13.41 ± 0.68bc | 20.40 ± 0.57a | 12.19 ± 0.31c | 14.79 ± 0.49b | $F(3,8) = 140.89$ $p$ value < 0.0001 |
| Threonine | 11.69 ± 0.66b | 15.88 ± 0.44a | 10.24 ± 0.29c | 12.41 ± 0.41b | $F(3,8) = 78.08$ $p$ value < 0.0001 |
| Tyrosine | 9.93 ± 0.79b | 18.00 ± 0.73a | 8.05 ± 0.25c | 10.35 ± 0.39b | $F(3,8) = 167.85$ $p$ value < 0.0001 |
| Valine | 13.67 ± 0.65bc | 17.49 ± 0.58a | 12.40 ± 0.27c | 14.89 ± 0.45b | $F(3,8) = 54.99$ $p$ value < 0.0001 |

#### *3.3.2. Observed dead insects*

Very few dead insects were observed under the flowering trees, with only two *Apis mellifera* individuals, two *Bombus terrestris* morphotype individuals, one *Lasioglossum marginatum*, and three Syrphidae (*Eupeodes latifasciatus* and *Episyrphys balteatus*) in a total of 80 h (20 h per tree species). Only one dead honeybee was found under *Tilia tomentosa*, showing no increased mortality for this late flowering *Tilia* species.

#### *3.3.3. Bumblebee survival*

Bumblebee workers fed exclusively on flowers of *T. cordata* or of *T. tomentosa* survived even well than bumblebees with no access to *Tilia* flowers (Figure 3). After 6 days per experiment, the bumblebee mortality ranged from 5 to 12 individuals (out of 30 for each experiment).

## 4. DISCUSSION

### 4.1. Are nectar and pollen offered in similar quantities and compositions?

Our four studied species differ in both the quantity and the composition of floral resources. *Tilia tomentosa* offered more flowers per cubic meter, more pollen grains per flower, and more nectar than the other studied *Tilia* species. Total sugar content in *Tilia* nectars ranged from 0.24 mg per flower (*T. tomentosa*) to 0.06–0.10 mg (three other species). Other authors reported similar sugar volumes and concentrations (Käpylä 1978; Pigott 1991; Krasenbrink et al. 1994; Baal et al. 1994; Gašić et al. 2014; Somme et al. 2016; Argoti 2016).

In pollen, a high nitrogen content, particularly with a protein content higher than 20% dw, is positively correlated with the development of bee larvae (Tasei and Aupinel 2008). Pollen from our studied *Tilia* trees contained more than 20% total amino acids and all

**Table II.** Minor sugar concentrations in nectar of the four studied *Tilia* species.

| Sugars | *T. cordata* | *T. platyphyllos* | *T. tomentosa* | *T. × europaea* | ANOVA |
|---|---|---|---|---|---|
| Mannose (ng/μL) | < LOD | < LOD | < LOD | < LOD | |
| Galactose (ng/μL) | 9.3 ± 15.3 | 6.3 ± 9.3 | < LOD | 2.7 ± 0 | $F(3,6) = 0.44$ $p$ value = 0.7337 |
| Myo-inositol (ng/μL) | 7.2 ± 11.7 | 1.0 ± 0.3 | 1.8 ± 0.5 | 0.6 ± 0 | $F(3,6) = 0.55$ $p$ value = 0.6689 |
| Raffinose (ng/μL) | < LOD | 0.07 ± 0.13 | 0.12 ± 0.21 | < LOD | $F(3,6) = 0.44$ $p$ value = 0.7354 |

*LOD* limit of detection

**Table III.** Extraction recoveries of SPE cartridges (concentrations in ng/mL) obtained for four solutions of different concentrations of nicotine spiked in *Impatiens* nectar passed through SPE cartridges ($m = 4$, $n = 1$, $k = 2$). The concentrations after SPE were calculated with the calibration curve.

| Nicotine spiked concentration before SPE | | Nicotine calculated concentration after SPE | Average nicotine concentration after SPE | Recovery (%) |
|---|---|---|---|---|
| 0.5 | 0.481 | 0.469 | 0.475 | 95.00 |
| 1.0 | 0.954 | 0.967 | 0.961 | 96.10 |
| 10.0 | 9.355 | 9.772 | 9.564 | 95.64 |
| 25.0 | 23.471 | 23.806 | 23.639 | 94.55 |

essential amino acids, which confirm their potential utility as pollen source (Fornoff et al. 2017). Furthermore, as pollen presented a balanced amino acid content, they are attractive to pollen-collecting insects (Somme et al. 2015). Pollen of *Tilia* trees could also be source of phytosterols as bumblebees collect pollen with both high amino acid and sterol contents (Vaudo et al. 2015).

### 4.2. Do the nectars contain mannose or nicotine?

No trace of mannose or nicotine has been detected in our nectar samples, whatever the studied species. Mannose and nicotine concentrations in the four species nectars were < 1.44 ng/μL and < 0.2 ng/mL respectively. The absence of mannose contradicted the results obtained by other authors

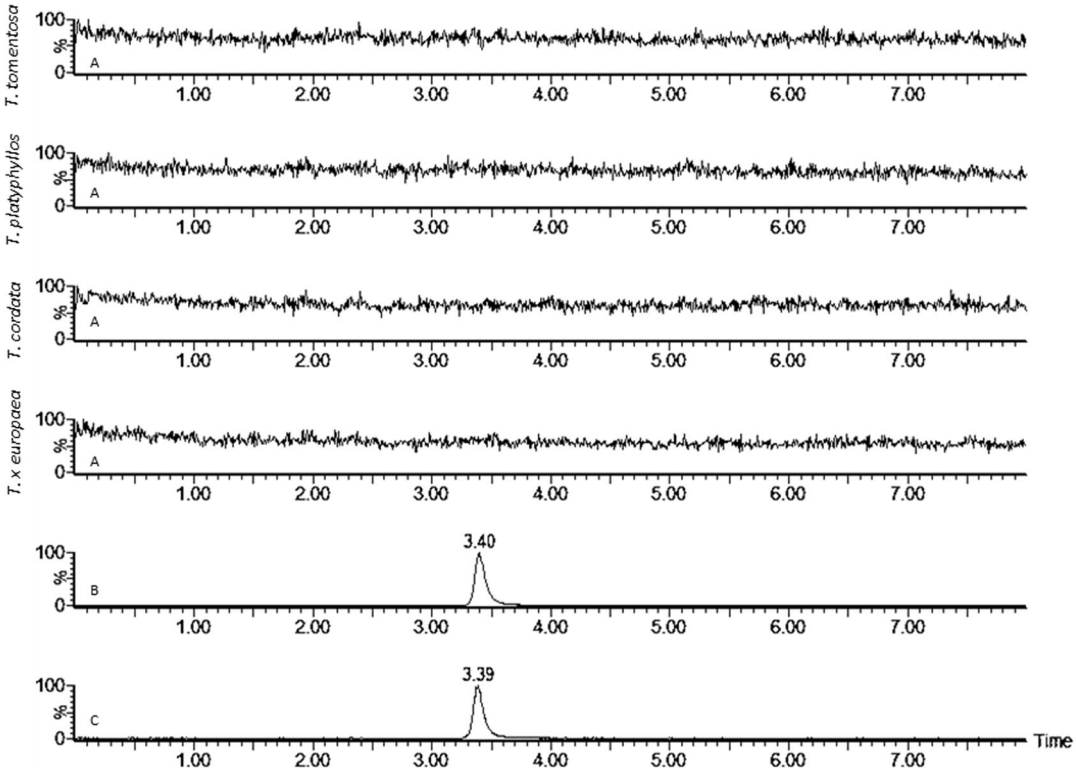


**Figure 2.** Chromatograms obtained after the SPE-LC-MS analyses of the nectars from the four studied species. **a** MRM for nicotine. **b** . MRM for D4-nicotine. **c** Total ion current. Chromatograms **b** and **c** are similar for the four species.

**Table IV.** Insect visitors observed on the four studied *Tilia* tree species at Louvain-la-Neuve, June and July 2016, on 70 sessions of 15 min in total ($N$ = total number of visitors observed per species).

| Insect visitors (%) | *T. cordata* | *T. platyphyllos* | *T. tomentosa* | *T.* × *europaea* |
|---|---|---|---|---|
| N visitors | 99 | 104 | 322 | 14 |
| Honeybees[a] | 79.8 | 55.8 | 87.3 | 28.6 |
| Bumblebees[b] | 6.1 | 27.9 | 0.6 | 71.4 |
| Syrphids[c] | 14.1 | 16.3 | 12.1 | 0 |

[a] Honeybees (Hymenoptera, Apidae) corresponded to *Apis mellifera*

[b] Bumblebees (Hymenoptera, Apidae) were represented by *Bombus terrestris* morphotype and *B. hypnorum* morphotype

[c] Syrphids (Diptera) included *Eupeodes latifasciatus*, *Episyrphus balteatus*, *Eristalis arbustorum*, *E. intricaria*, *E. tenax*, *Helophilus trivittatus*, and *Syritta pipiens*

(Madel 1977; Argoti 2016). Several studies have stated that mannose is toxic and present in the nectar without chemically identifying its presence in *Tilia* nectar (Wykes 1952; Pigott 1991; Pawlikowski 2010). Madel (1977) found mannose in the nectar of *T. tomentosa* using paper chromatography. However, based on more precise techniques, such as gas chromatrography, Baal et al. (1994) or Gašić et al. (2014) failed to detect mannose in the nectar or even in other parts of the flowers.

The assumption that *Tilia* nectar contains mannose potentially originates from a time when modern analytical methods had not yet been used (Argoti 2016; VKM 2017).

Contrarily to our results, trace amounts of nicotine have been indicated to occur in *Tilia* nectar (Naef et al. 2004). Nevertheless, Singaravelan et al. (2006) concluded that honeybees can cope with naturally occurring concentrations of nicotine, even when consumed in large quantities (up to 50 ng/μL). Moreover, bumblebees may even recover if fed with nectar from *T. tomentosa* tree shortly after falling to the ground (Surholt et al. 1992). These studies question the toxicity for bumblebees of secondary metabolites in *Tilia* nectar. We can therefore conclude that no toxic compounds have been yet detected in *Tilia* nectar. In the same order, toxicity of the pollen or of the honeydew seems unlikely (VKM 2017). However, toxicity tests of the volatile secondary metabolites found in nectar are still lacking (VKM 2017).

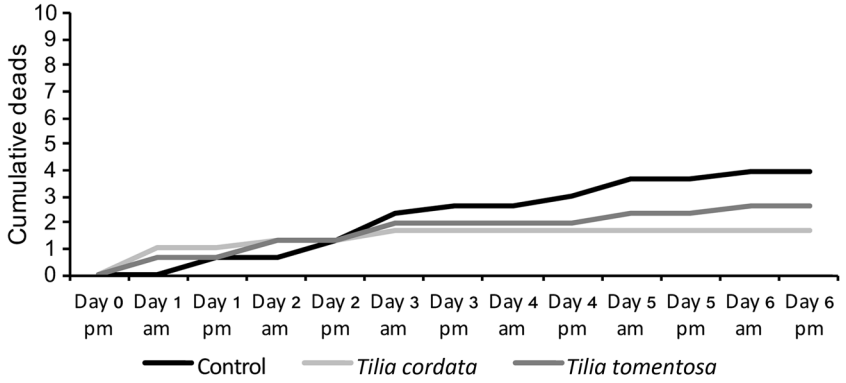


**Figure 3.** Cumulative dead *Bombus* over ten individuals under each of the three mosquito nets and from three different hives (three experiments): control with bumblebees fed with pollen and Biogluc ad libitum and access to branches of *Tilia* spp. without any flowers (black line), with *Tilia cordata* flowers (pale gray line), and with *Tilia tomentosa* flowers (dark gray line).

### 4.3. Do the different *Tilia* species attract similar insect visitors?

As in other studies, even in a few hours, we observed diverse visitors on *Tilia* species, including honeybees, bumblebees, and syrphids. Even if their numbers and proportions depend on the activity of the surrounding apiaries, honeybees are considered numerous and constant on *Tilia* trees (Knuth 1908; Anderson 1976; Pigott 1991; Illies and Mühlen 2007; Pawlikowski 2010). Our observed bumblebees, *Bombus terrestris* and *B. hypnorum*, have already been determined in other sites (Knuth 1908; Pigott 1991; Illies and Mühlen 2007; Pawlikowski 2010). Bumblebee fidelity to *Tilia* pollen has been assessed as much as 70% of the bee pollen loads contained *Tilia* pollen (Weryszko-Chmielewska and Sadowka 2010).

### 4.4. Do bumblebees present any mortality?

We did not detect any particular mortality among the bumblebee workers fed only on *Tilia* flowers. In fact, several authors did not mention any death during their observations (Knuth 1908; Free 1970) or only reported few bees crawling (Illies and Mühlen 2007). In our study site, spring and summer climatic conditions in 2016 (May to August) were considered normal with a mean temperature about 16 °C, and after a rainy spring (78.3 mm in May), summer months did not present any pluviometry deficit (55 mm in June and July; Royal Meteorological Institute Belgium 2016). We were not able to test *in natura* the mortality of bees under water deficit evoked by Crane (1977).

Bees are prone to starvation if they fail to find sufficient nectar resources during a foraging bout (Baal et al. 1994; Surholt and Baal 1995; Illies 2016; VKM 2017; Koch and Stevenson 2017). Illies and Mühlen (2007) observed that the number of dead bumblebees increased throughout the season and reached the maximum at the end of the flowering period of *T. tomentosa*. As 3–4 mg of nectar can be produced by a single flower, and this amount will be replaced on successive days (Anderson 1976), it seems curious that such high resource can induce bee starvation. *Tilia* trees produce more nectar than the other planted urban trees (Somme et al. 2016). In consequence, more detailed monitoring of nectar availability related to bee death is needed in order to assess this hypothesis.

A last hypothesis posits a natural death of bees (VKM 2017). *Tilia* trees are flowering in summer, coinciding with a peak in the development of the colonies. As bumblebee individuals have a short lifetime expectancy, the mortality is high. Mühlen et al. (Mühlen et al. 1992, 1994) found that the majority of the dead bumblebees were workers, which can confirm this hypothesis. As bees tend to accumulate to forage on *Tilia* trees, the deaths observed might be a result of the patchy distribution of workers (VKM 2017). As *Tilia* trees are often planted in parks or paved sidewalks with short vegetation or bare ground, dead bees are more easily visible there than under other vegetation. Indeed, if comparable floral resource hotspots exist, they should be associated with deaths to a similar extent. To our knowledge, such comparisons of numbers of dead bees are still lacking. More information about the natural death hypothesis is needed.

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## AUTHOR CONTRIBUTIONS

ALJ, MQ, JQL, and MFH conceived this research and designed the experiments; WS, MQ, PO, and LM performed experiments and analysis; ALJ, MQ, JQL, and MFH wrote the paper and participated in the revisions of it. All authors contributed critically to the drafts and approved the final manuscript.

## COMPLIANCE WITH ETHICAL STANDARDS

**Conflict of interest** The authors declare that they have no conflict of interest.

**_Tilia_: ressources toxiques ou précieuses pour les pollinisateurs?**

**tilleuls / _Tilia tomentosa_ / nicotine / mannose / abeilles / nectar / pollen**

**Lindenbäume: Giftige oder wertvolle Resourcen für Bestäuber?**

**Lindenbäume / _Tilia tomentosa_ / Nikotin/ Mannose / Bienen / Nektar / Pollen**

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