ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

# $LD_{50}$ VALUES MAY BE MISLEADING PREDICTORS OF NEONICOTINOID TOXICITY ACROSS DIFFERENT BEE SPECIES

## Neonikotinoidlerin Zehir Etkilerini Belirlemede $LD_{50}$ Değerleri Farklı Arı Türleri İçin Yanıltıcı Bir Öngösterge Olabilir

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**ABSTRACT**

**The importance of not only honey bees (*Apis mellifera*) but also other non-managed bee species and their pollination services has come to light with their recently reported declines. One contributing factor in these declines is thought to be sub-lethal exposure to neonicotinoid insecticides such as thiacloprid. However, current government regulatory agencies do not require the assessment of insecticide toxicity on bee species other than the honey bee, even though previous studies have demonstrated that sensitivity to insecticides is not likely to be generalizable from honey bees to non-managed bee species. Replicating standardized protocols and testing five different doses of thiacloprid on individual caged bees, we assessed the acute contact toxicity by calculating mortality and the lethal dose ($LD_{50}$) value for three bee species with different life history traits: *Apis mellifera*, *Bombus terrestris*, and *Osmia bicornis*. We found that *Apis mellifera* and *Osmia bicornis* had significantly higher mortality in comparison to *Bombus terrestris*, but there was no dose-dependent response for any of the three bee species. Bee size and sex were also not useful predictors of thiacloprid toxicity. These results suggest that solely relying on $LD_{50}$ values, especially when they do not produce a dose-dependent response, may be misleading when assessing insecticide toxicity risk for honey bees and other non-managed bee species.**

**Keywords:** Neonicotinoid, Thiacloprid, Bee health, Mortality, Toxicity

**ÖZ**

**Son yapılan kayıp raporları ile sadece bal arıları değil diğer yabani arılar ve onların yaptığı tozlaşma hizmeti gündeme gelmiş oldu. Bu kayıpların oluşmasında önemli faktörlerden biri örneğin thiacloprid gibi neonikotinoid böcek öldürücülerin ölümcül etkinin altındaki dozları düşünülmektedir. Daha önce yapılan çalışmalar göstermiştirki böcek öldürücülere karşı duyarlılığı bal arıları üzerinde yapılan çalışmaları kullanarak yabani arılar için genelleştirmek doğru olmaz. Gerçi güncel devlet düzenleme kurumları bal arısı dışında diğer arılar üzerinde böcek öldürücüler ile ilgili değerlendirmeyi gerekli görmez. Kafese konulmuş her bir arı üzerinde thiacloprid'in beş farklı dozunu test ve standart protokolü tekrar ederek farklı yaşam karakterlerine sahip üç farklı arı türü için ani temas ile zehirlenmeyi ölüm oranlarını hesaplayarak ve ölümcül doz ($LD_{50}$) değerlerini kullanarak belirledik. Bu**

**çalışma ile *Apis mellifera* ve *Osmia bicornis* türlerinde *Bombus terrestris*'e göre ciddi derecede yüksek arı ölümleri tespit ettik. Fakat üç farklı arı türü için doza bağlı bir reaksiyon görülmemiştir. Arı büyüklüğü ve cinsiyet thiacloprid zehirlenmesi için yararlı bir öngösterge değildir. Bu sonuçlara göre doza bağlı bir reaksiyon üretilmeden tamamen $LD_{50}$ değerlerine güvenmek, bal arılarında ve yabani arı türlerinde böcek öldrücülerin zehir seviyesini belirlemede yanıltıcı olabilir.**

**Anahtar Kelimeler:** Neonicotinoid, Thiacloprid, Arı sağlığı, Ölüm oranı, Zehirlilik

## GENİŞLETİLMİŞ ÖZET

**Amaç**: Son yapılan kayıp raporları ile sadece bal arıları değil diğer yabani arılar ve onların yaptığı tozlaşma hizmeti gündeme gelmiş oldu. Bu kayıpların oluşmasında önemli faktörlerden biri örneğin thiacloprid gibi neonikotinoid gibi böcek öldürücülerin ölümcül etkinin altındaki dozları düşünülmektedir. Daha önce yapılan çalışmalar göstermiştirki böcek öldürücülere karşı duyarlılığı bal arıları üzerinde yapılan çalışmaları kullanarak yabani arılar için genelleştirmek doğru olmaz. Gerçi güncel devlet düzenleme kurumları bal arısı dışında diğer arılar üzerinde böcek öldürücüler ile ilgili değerlendirmeyi gerekli görmez.

Bu nedenle bu çalışmanın amacı bal arılarındaki thiacloprid zehir seviyesinin diğer arılar için genelleme yapılıp yapılamayacağıdır.

**Gereç ve Yöntem:** Bu çalışma Almanya Martin Luther Üniversitesi Genel Zooloji bölümünde Arı laboratuvarında yapılmıştır. Çalışma 2014 yılı Haziran-Ağustos arasında 24 °C de laboratuvar koşullarında yapılmıştır.

*Osmia bicornis* kozaları önceki yıl kültüre alınmış *Phragmites köklerini içeren* suni yuva kutularından hasad edilmiştir. Kozalar köklerden alınıp 4 °C ihtiyaç olana kadar tutulmuştur. Bombus terrestris yuvaları ise ticari olarak KOPPERT Deutschland'den satın alınmış, üç yuva 24 °C laboratuvarda tutulmuş ve ağızdan sukroz ve polen ile beslenmiştir. Yeni çıkan dişi işçi arılar üç yuvadan böcek vakumu kullanılarak tesadüfi olarak toplanmıştır. Her gurup için en az 30 işçi arı kullanılmıştır.

Yeni çıkan *Apis mellifera* arılarını tedarik etmek için 3 farklı kökenli koloniden yavru çerçeveleri alındı ve 35 °C inkübatörde tutulurken gece ergin arı olarak çıkmışlardır. Bu çerçevelerden (< 24 saat) deneme için çıkan arılar tesadüfi olarak alınmıştır. Her bir deneme gurubu için en az 30 işçi kullanılmıştır. Daha sonra *Apis mellifera*'da zehirlenme araştırmaları için standart kılavuz takip edilmiştir (Medrzycki et al. 2013). Kafese konulmuş her bir arı üzerinde thiacloprid'in beş farklı dozunu test ve standart protokolü tekrar ederek farklı yaşam karakterlerine sahip üç farklı arı türü için ani temas ile zehirlenmeyi, ölüm oranlarını hesaplayarak ve ölümcül doz ($LD_{50}$) değerlerini kullanarak belirledik.

**Bulgular:** Bu çalışmada *Apis mellifera* ve *Osmia bicornis* türlerinde *Bombus terrestris*'e göre ciddi derecede yüksek arı ölümleri tespit ettik. Ek olarak işlem görmeyen kontrol arıları böcek öldürücüler ile muamale edilen arılar göre çiddi derecede yüksek yaşama seviyesi göstermiştir. Fakat üç farklı arı türü için doza bağlı bir reaksiyon görülmemiştir.

*Apis mellifera* and *Osmia bicornis* türleri *Bombus terrestris*'e göre thiacloprid'e oransal olarak daha yüksek ani temas hassasiyeti göstermiştir. Gerçi *A. mellifera* ve *O. bicornis* vücut büyüklüğü olarak benzer fakat oldukça farklı $LD_{50}$ değerlerine sahiptir.

**Sonuç:** Arı büyüklüğü ve cinsiyet thiacloprid zehirlenmesi için yararlı bir öngösterge değildir. Bu sonuçlara göre doza bağlı bir reaksiyon üretilmeden tamamen $LD_{50}$ değerlerine güvenmek, bal arılarında ve yabani arı türlerinde böcek öldürücülerin zehir seviyesini belirlemede yanıltıcı olabilir.

Bu yüzden karar alıcılardan sadece doğal ortamda böcek öldürücüler için uzun süreli hassas testlerin ve öldürücü dozun altındaki etkisinin uzun süreli etkilerinin yapılmasının tavsiye edilmesi değil aynı zamanda farklı arı türleri üzerinde böcek öldürücülerin zehir seviyesini belirlemede $LD_{50}$ sayılarının değerlendirmesinin kullanılması tekrar düşünülebilir. Hatta tarım ilaçlarının zehir seviyesini rakamsal olarak değerlendirmede standart ölçüt olarak düşünülebilir.

## INTRODUCTION

With intensified agricultural production required to meet growing food demands around the world, we rely upon the pollination service of bees to increase per capita agricultural output (Winfree et al., 2011). Pollinators not only increase crop yields, they also increase the quality of produce as well (Aizen and Harder, 2009; Klein et al., 2007). Not only honey bees, but also wild native bees are important for the pollination of agricultural crops (Brittain et al., 2013; Giannini et al., 2014; Garibaldi et al., 2016); their combined pollination service has been economically valued at 15 billion USD in the United States, 11.40 billion USD (43 billion Brazilian reais) in Brazil alone and 202 billion USD on a global scale (Calderone, 2012; Gallai et al., 2009; Hein, 2009; Wolowski et al., 2019). Bees and especially solitary bees provide an essential ecosystem service of pollination that plays a major role in sustaining biodiversity of primary forests and other ecosystems (Bawa, 1990).

Despite our dependence on bees for their pollination services and maintaining ecosystem stability, there is a consistent and recent decline of both managed (*e.g.* honey bees and some bumble bees) and non-managed (wild) bee populations in many northern temperate regions of the world (Biesmeijer et al., 2006; Brown and Paxton, 2009; Freitas et al., 2009; Potts et al., 2010; Ricketts et al., 2008). Since the first report of bee declines, multiple stressors have been identified as playing possible roles, including parasites, insecticides, loss of foraging habitat, and loss of nesting habitat (Potts et al., 2010). There are numerous studies demonstrating that sub-lethal exposure to insecticides, and in particular exposure to neonicotinoids, is likely one of the factors impacting bee health. Although not linked to outright increases in mortality based on lab studies, sub-lethal exposure to neonicotinoids alone results in impaired navigation, a loss of fecundity, premature mortality, and in the case of honey bees, causes a loss of colony strength in terms of brood production (Blacquière et al., 2012; Doublet et al., 2015; Fischer et al., 2014; Henry et al., 2012; Jin et al., 2015; Krupke et al., 2012; Rundlöf et al., 2015; Sandrock et al., 2014a; Sandrock et al., 2014b; van der Sluijs et al., 2013; Whitehorn et al., 2012) and reduction of social interaction as shown to eusocial stingless bees (Boff et al., 2018). Neonicotinoid insecticides, the most common of which include imidacloprid, acetamiprid, thiacloprid and thiamethoxam, deserve special attention because they are known to have varying toxicity levels (Sanchez-Bayo and Goka, 2014), even though they all mechanistically act in a similar manner as an antagonist of insect nicotinic acetylcholine receptors (nAChR) (Elbert et al., 2008; Matsuda et al., 2001). There is difficulty in generalizing neonicotinoid toxicity, despite the numerous studies demonstrating sub-lethal effects, so this has raised the question if the standard acute honey bee contact toxicological assays can be used as a reliable indicator of the potential risks these insecticides pose to other wild bee species (Decourtye et al., 2013).

There are over 20.000 bee species that live in diverse habitats and vary vastly in life-history and morphological traits (Michener, 2000); all of these factors are likely to affect the route of insecticide exposure and the subsequent insecticide toxicity level for each bee species. Despite this variability across bee species, the honeybee alone serves as the model for insecticide toxicity testing. Regulation agencies do not require toxicity testing on other non-managed bee species and, although there are advantages to using the commercially available honey bee due to practical considerations and the important role they play for their pollination services in several crops (Hein, 2009), recent evidence suggests that non-managed bees are much more sensitive to insecticide exposure in a field setting (Rundlöf et al., 2015; Gradish et al., 2018). This finding suggests that other non-honey bee species need to be considered in their own right when assessing the risk of insecticide use; they should not be neglected when assessing toxicity effects of apesticide on non-target insect species (Park et al., 2015).

Addressing this concern, several pesticides have been tested in ecotoxicological studies across bee species in order to understand their toxicity on not only the honey bee (Cresswell et al., 2012; Iwasa et al., 2004) but also on bumble bees (Laycock et al., 2012; Scott-Dupree et al., 2009; Whitehorn et al., 2012), leafcutter bees (Scott-Dupree et al., 2009), and stingless bees (Boff et al., 2018). Results are consistent in that insecticide exposure increases mortality rates, though rates vary across bee species. But comparative studies under controlled laboratory conditions, seeking to draw generalities regarding bee insecticide toxicity, are relatively rare (Blacquière et al., 2012). Previous studies using metadata have shown that the level of pesticide toxicity for a particular bee species is dependent upon the kind of insecticide class, age of the bee, and route of exposure (Arena and Sgolastra, 2014).

But in general, the toxicity of insecticides across different bee species based on lethal dose 50 ($LD_{50}$) values in a controlled laboratory setting is variable.

We have therefore chosen three different representative bee species: *Apis mellifera*, *Bombus terrestris*, and *Osmia bicornis* that vary drastically in life history traits, in their level of sociality, individual body size and morphology, to assess the toxicity of the neonicotinoid thiacloprid. Our goal was to replicate the standard toxicity testing established by government guidelines, which includes assessing bee mortality after acute contact exposure to establish $LD_{50}$ values for each bee species. We tested five different thiacloprid concentrations–and we measured bee size to determine if it could be used as a reliable predictor of toxicity. We also accounted for sex differences in *Osmia bicornis*. Typically, only female honey bee worker are used to assess insecticide effects; however, in solitary bees the sex ratio and male survival is likely more equally weighted in terms of population stability (Seidelmann, 2014) and therefore male survival is also critical for population viability. From these assessments, we then determined if honeybee thiacloprid toxicity levels could be generalizable to other bee species.

## MATERIAL AND METHODS

The study took place in the Bee Lab of the Department of General Zoology in the Martin Luther University Halle-Wittenberg, in Halle Germany. The experiment was conducted in laboratory conditions at 24°C during summer (June – August) of 2014.

### *Collection of bees*

*Osmia bicornis* cocoons were harvested from artificial nest boxes containing *Phragmites* stems, which were cultured in Halle (Saale) the previous year. Cocoons from the stems were stored in the refrigerator (4°C) until needed. The cocoons were sexed based on size and this was then verified by carefully opening the cocoon to check whether the bee had white hair above the clypeus (which indicated it was a male bee). Each intact cocoon was then placed into an Eppendorf tube (2 mL) with holes for aeration; tubes were held in an incubator at 24°C and 70% relative humidity, until they emerged. At least 30 individuals were collected per sex per treatment group.

Three *Bombus terrestris* nests were commercially purchased from Koppert Deutschland GmbH. All three nests were kept at 24°C inside the laboratory and were fed sucrose solution and pollen *ad libitum*. Freshly emerged female worker bees were collected randomly from all three nests using an insect vacuum. At least 30 worker bees were collected per treatment group.

To obtain newly emerged *Apis mellifera* bees, we took brood frame from 3 different source colonies and placed frames in an incubator held at 35°C, from which adults hatched out overnight. From these frames we randomly took freshly emerged (< 24 h) workers for experimentation. At least 30 workers were harvested per treatment group.

### *Insecticide preparation and application*

We followed the guidelines of standard methods for toxicology research to test acute topical insecticide toxicity on *Apis mellifera* (Medrzycki et al., 2013). First, we prepared a total of five thiacloprid insecticide doses relative to the already established acute contact toxicity $LD_{50}$ value (38,83 µg/bee, our 100% dose) of honey bees (FERA, 2013). We made the following thiacloprid concentrations using acetone as solvent: 125% (48,54 µg/bee), 25% (9,71 µg/bee), 2% (0,79 µg/bee) and 1% (0,39 µg/bee) of the honeybee $LD_{50}$ value along with 0% (0 µg/bee), which served as a control to account for the effects of acetone (Di Prisco et al., 2013). The 2% sub-lethal dose represents a realistic field exposure dose (Smodiš Škerl et al., 2009) and a 1% dose represents what is considered to be sub-lethal exposure for the honeybee (Vidau et al., 2011). The 25% dose was chosen to be scaled as one fourth less than the $LD_{50}$ value but greater than the known sub-lethal exposure values of 1%. The 125% dose was chosen as a positive control, a value that was certain to cause some sort of mortality after 48 hours. Right before application, all thiacloprid insecticide dosages were vortexed vigorously for at least 1 min to force the thiacloprid into solution.

Each individual from the three species was transferred one at a time to a honeybee queen marking cage to immobilize the bee, whereupon it received a 1 µl topical insecticide application on the back of the thorax using a micropipette. Immediately after the application, individual bees were transferred to metal cages (10 × 10 × 6 cm) where they remained individually and were fed *ad libitum* 50% sucrose solution with 1% Provita Bee protein supplement using a 1,5 mL Eppendorf® tube with 3

holes as feeder to facilitate feeding. Cages were maintained in a temperature-controlled laboratory (24°C) with exposure to natural light from the window. Bees were observed every 24 h for a total of five days and were considered dead if they had stopped moving, even after shaking the cage. All the dead bees during the census were recorded, removed from their cage, and stored at -20°C. At any one trial there were up to 17 bees tested at a time individually and up to 4 trials were carried out consecutively until there was roughly a sample size of 30 bees per species, per sex, per treatment group (see total sample size in Table 1). After 5 days all live bees were freeze-killed at -20°C. We measured the intertegular span of each bee using a digital camera (Olympus DP21) attached to stereomicroscope (Olympus SZX7) (20x) and this was used as an indicator of body size. To standardize measurements in µm using cellSens V1.3 (Olympus) software, a straight-line segment between the compound eyes had to pass through all three ocelli on top of the bee head to insure the measurement was consistent (Cane, 1987).

***Statistical Analyses***

A Cox regression analysis was performed as a survival analysis across the 5 days of the experiment. The hazard ratio, calculated by the cox regression, was considered as the dependent variable, the insecticide dose, size, sex, and species of bee were considered as the fixed factors, and trial was considered as a random effect. The hazard ratio is defined as the probability that death will occur at a given time, and it is calculated by dividing the probability of the treatment group by the probability of the control group. This hazard ratio represents the instantaneous death rate for an individual who has already survived to this given time point. The insecticide dose of 0,00 µg/bee for the insecticide dose factor and *A. mellifera* for the species factor served as the null model, respectively, for the cox regression analysis (shown in Table 1). Further analysis using a Cox regression was carried out to determine if there was a relationship between size and mortality within each bee species. In addition, Cox regression followed by a Wald test was used to determine if there was a significant difference in survival between male and female *O. bicornis*. Post hoc analysis included a Tukey test for multiple comparisons across the hazard ratios derived from the Cox regressions. Log dose–response curves were used for the determination of $LD_{50}$ values according to probit analysis (Finney, 1952). Then a generalized linear model (GLM) logistic regression on the original dataset was conducted to analyze if there was a dose-dependent response, where 48-hour mortality served as the dependent variable, to match standard conventions to assess acute mortality, and the dose administered was considered as the independent variable. All statistical analyses were performed in R studio v. 2.15.2 (R Development Team, 2008).

**Table 1. Results of the Cox regression.** Results of the survival analysis: mortality (hazard ratio) as the dependent variable and insecticide treatment (dose) and species as fixed factors. This was done to verify that mortality was significantly higher than the control treatment of 0,00 µg/bee. The beta coefficients represent the magnitude of change in the hazard ratio based on the given factor in the table below with Exp representing the exponent of the beta coefficient and SE representing the standard error of the beta coefficient. Asterisks indicate significant p-values at the alpha = 0.05 level.

| Factor | Beta Coefficient | Exp of the Beta coeff | SE of the Beta coeff | Z-score | P-value |
|---|---|---|---|---|---|
| Insecticide dose | | | | | |
| 0,00 (µg/bee) (null model) | 4,11e-15 | 1,000 | 0,109 | 0,00 | 1,00 |
| 0,39 (µg/bee) | 0,8827 | 2,4175 | 0,1815 | 4,863 | < 0,001* |
| 0,79 (µg/bee) | 0,9929 | 2,6992 | 0,1805 | 5,502 | < 0,001* |
| 9,71 (µg/bee) | 0,9462 | 2,5759 | 0,1790 | 5,286 | < 0,001* |
| 38,83 (µg/bee) | 1,0075 | 2,7388 | 0,1797 | 5,607 | < 0,001* |
| 48,54 (µg/bee) | 0,9215 | 2,5131 | 0,1790 | 5,147 | < 0,001* |
| Species | | | | | |
| *A. mellifera* (null model) | 4,11e-15 | 1,000 | 0,109 | 0,00 | 1,00 |
| *B. terrestris* | -0,814 | 0,443 | 0,136 | -5,973 | < 0,001* |
| *O. bicornis* | 0,082 | 1,085 | 0,116 | 0,706 | 0,48 |

## RESULTS

Overall, *A. mellifera* and *O. bicornis* exhibited significantly higher mortality in comparison to *B. terrestris* across the 5 days of the survival experiment (P<0,001). In addition, the untreated control bees had significantly higher survival in comparison to the insecticide treated bees (P<0,001, Table 1, Fig. 1).

There was a strong trend, of *O. bicornis* males dying faster than females (Wald test: $X^2_{1,303}$ = 3,66, P = 0,056, Fig. 2). Additionally, there was a significant increase in mortality in the treated bees in comparison to the control bees, except for *B. terrestris*. For all three bee species, there was no differences in mortality within species over the five days across the insecticide doses administered, as indicated by the hazard ratios for *A. mellifera* (Fig. 3a), *B. terrestris* (Fig. 3b), and *O. bicornis* (Fig. 3c, Table 1). This result is independent of body size within a species as there is no significant relationship between size and mortality for *A. mellifera* (Cox regression: $r^2$ = 0,005, N = 219, P = 0,30), *B. terrestris* ($r^2$ = 0,013, N = 190, P = 0,12) or *O. bicornis* ($r^2$ = 0,002, N = 304, P = 0,48).

The $LD_{50}$ calculated for *A. mellifera* is 11,42 µg/bee, 9566,31 µg/bee for *B. terrestris*, and 4862,98 µg/bee for *O. bicornis* (Table 2). However, there was no dose-dependent response for *A. mellifera* (logistic regression: N = 182, slope = -0,0045, P = 0,51), *B. terrestris* (N = 157, slope = 0,0077, P = 0,34), and *O. bicornis* (N = 252, slope = -0,0041, P = 0,54, Fig. 4)

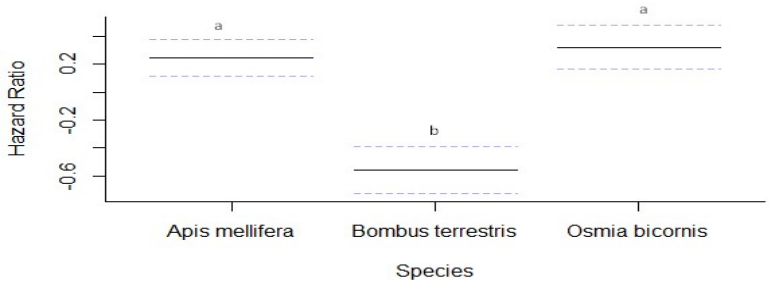



**Figure 1.** Mortality of the three bee species (*Apis mellifera*, *Bombus terrestris*, and *Osmia bicornis*) across the duration of the five day experiment plotted in terms of a hazard ratio defined as the instantaneous risk of death. Each line represents the mean (± SE) with letters denoting significant differences at the 0.05 alpha level resulting from a Tukey post hoc multiple comparisons test.

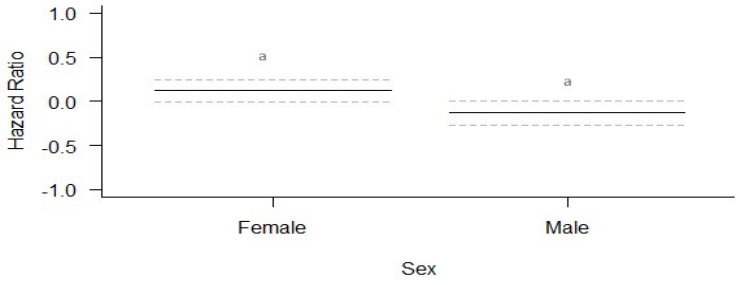
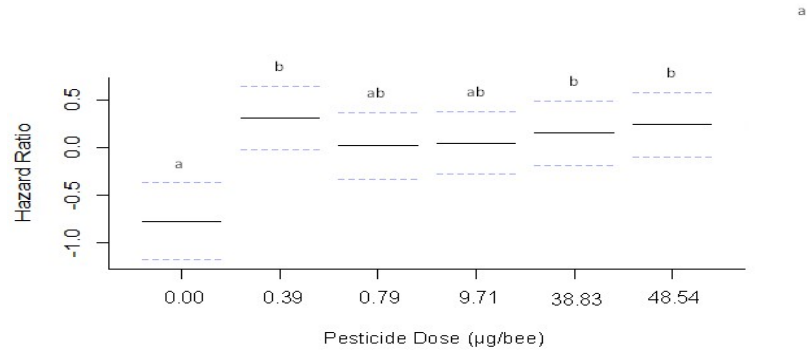
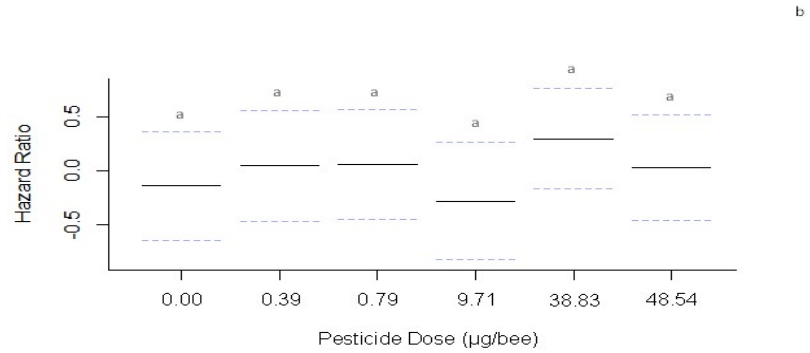


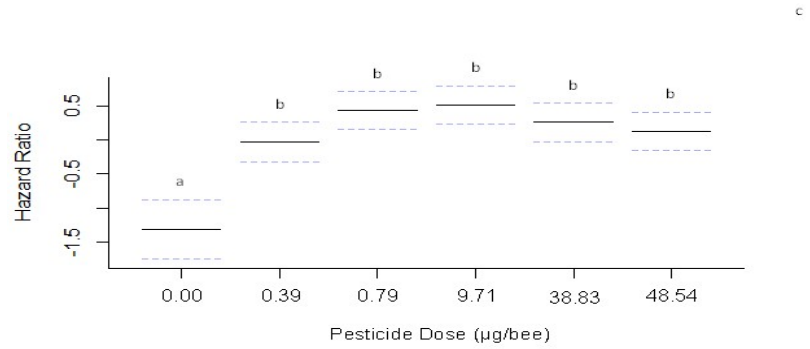

**Figure 2.** Mortality of male and female *Osmia bicornis* bees across the duration of the five day experiment represented as hazard ratios defined as the instantaneous risk of death. Each line represents the mean (± SE), with letters denoting significant differences at the 0.05 alpha level.




*Apis mellifera*




*Bombus terrestris*




*Osmia bicornis*

**Figure 3.** Mortality of *Apis mellifera* (a), *Bombus terrestris* (b), and *Osmia bicornis* (c) represented as hazard ratio defined as the instantaneous risk of death calculated from the five day survival experiment. Five different pesticide doses are presented from lowest to highest which includes a sublethal dose of 1/100$^{th}$ the LD50 value, 0.39 µg/bee, a field exposure equivalent of 1/50$^{th}$ of the LD50 value, 0.79 µg/bee, 1/4$^{th}$ of the LD50 value 9.71 µg/bee, the LD50 value of 38.83 µg/bee, 1/4$^{th}$ increase of the LD50 value, 48.54 µg/bee, and controls treated with 0.0 µg/bee. Each line represents the mean (± SE) with letters denoting significant differences at the 0.05 alpha level resulting from a Tukey post hoc multiple comparisons test.

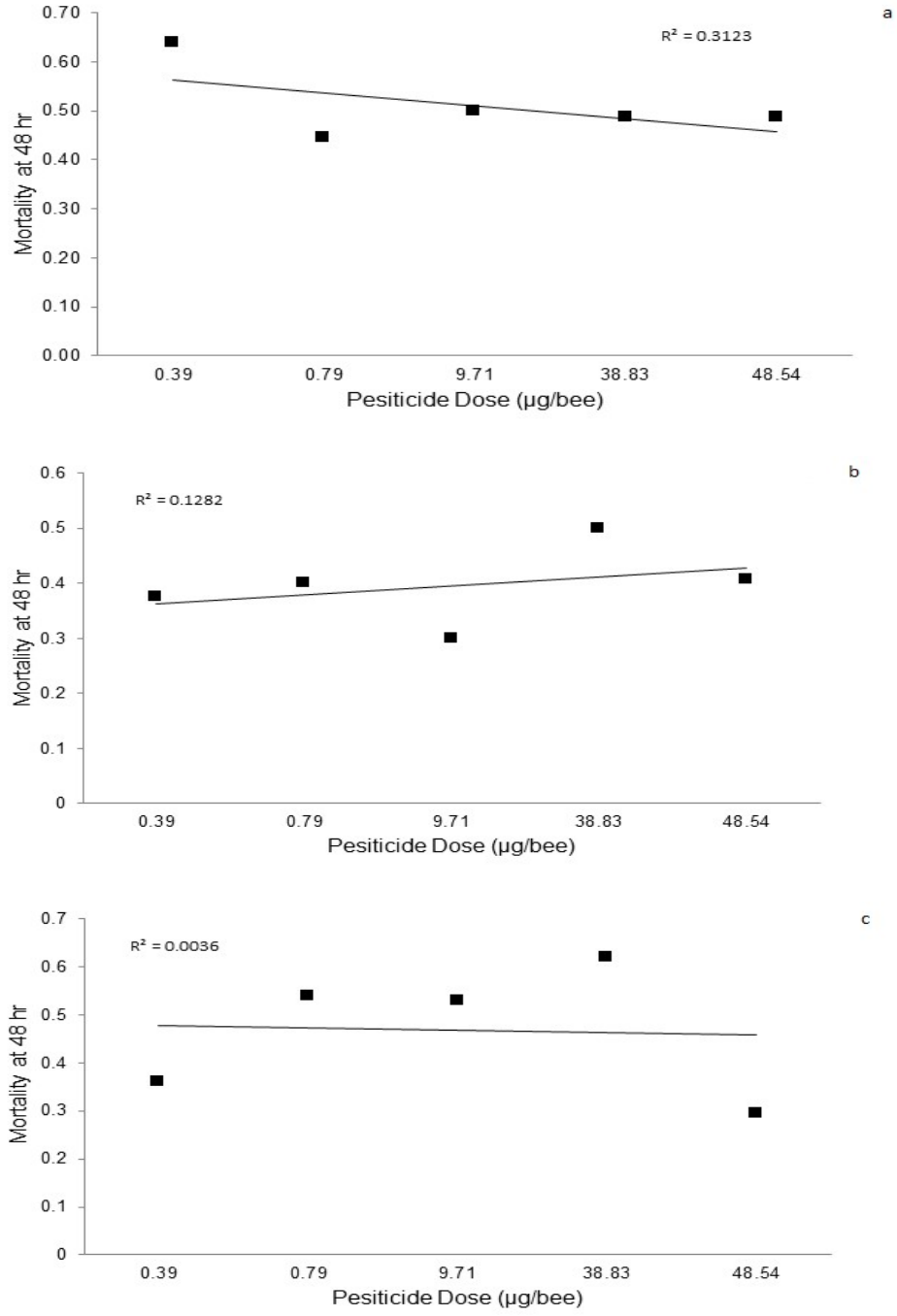



**Figure 4.** The $LD_{50}$ dose dependent curves for *Apis mellifera* (a) *Bombus terrestris* (b), and *Osmia bicornis* (c). Based on 48 hour mortality after the start of the survival experiment.

**Table 2. $LD_{50}$ Calculations.** The $LD_{50}$ values back calculated in original units across the three bee species calculated from a log-dose response curve using an Abbot transformation and probit analysis. SE stands for standard error of the slope estimate.

| Bee Species | Sample size (N) | $LD_{50}$ (µg/bee) | Slope (b) | SE (slope) | $\chi^2$ |
|---|---|---|---|---|---|
| *Apis mellifera* | 182 | 1,42 | -0,09306 | 0,10807 | 0,74 |
| *Bombus terrestris* | 157 | 9566,31 | 0,0779 | 0,1171 | 0,44 |
| *Osmia bicornis* | 252 | 4862,98 | 0,02718 | 0,09197 | 0,087 |

## DISCUSSION

*Apis mellifera* and *Osmia bicornis* have relatively higher acute contact sensitivity to thiacloprid than *Bombus terrestris*. Although *A. mellifera* and *O. bicornis* are similar in size, they have widely different $LD_{50}$ values. Our results based on $LD_{50}$ values are in agreement with previous studies that show *A. mellifera* tends to be relatively more sensitive to contact insecticide exposure when comparing across many bee species (Arena and Sgolastra, 2014; Del Sarto et al., 2014). Although both of these meta-analyses (Arena and Sgolastra, 2014; Del Sarto et al., 2014) demonstrate a large range in sensitivity to insecticides and that generally *Apis* bees are more sensitive to pesticides than *Osmia* bees, we demonstrate here based on mortality that *O. bicornis* is just as sensitive to thiacloprid as *A. mellifera*. This finding therefore highlights the need for more empirical tests to be conducted under controlled laboratory conditions so that accurate comparisons of insecticide sensitivity can be made across different bee species, as the toxicity of insecticides is a result of a set of complex interactions.

Heard et al. (2017) [references listesinde yok] tested a range of pesticides on *O. bicornis* and *B. terrestris* in the lab and compared their toxicity with *A. mellifera*, they found relatively consistent results, but thiacloprid was not included in their study and they tested pesticides via oral exposure, which tends to have more consistent effects in comparison to contact exposure. In this study they also found a few exceptions to the reproducibility of the pesticide tests across species when a time component was considered. Gradish et al. (2018), when comparing pesticide exposure across bee species, mentions that bumble bees are more sensitive, but they largely refer to the queen bumble bee, which overwinters in the soil and suspect that the route of exposure the pesticides may be different in comparison to foraging honey and bumble bees. Our mortality results support the findings that in well controlled laboratory tests bumble bees with contact exposure to pesticides are generally less sensitive in comparison to honey bees (Sanchez-Bayo and Goka, 2014).

The $LD_{50}$ values we calculated are not indicative of bee sensitivity to insecticide exposure and this is likely due to the fact that we did not find a dose-dependent response across any of the three bee species. Our non-dose-dependent curves are responsible for the large variance in the predicted $LD_{50}$ values for each of the three bee species. This finding may not only be dependent upon pesticide type, but also the route of exposure (Badawy et al., 2015). In addition, each neonicotinoid can have dramatically different relative toxicological effects across bee species (Badawy et al., 2015; Biddinger et al., 2013; Iwasa et al., 2004; Valdovinos-Nunez et al., 2009), despite each neonicotinoid insecticide belonging to the same family, which operate in a mechanistically similar manner (Tomizawa and Casida, 2005). However, despite the differences resulting from the factors mentioned above, we would interpret the relative lower sensitivity to thiacloprid, as indicated by the much higher $LD_{50}$ values in our study, with caution, because it contradicts the relatively high sensitivity to sublethal insecticide exposure of *Bombus* or *Osmia* bees in a more natural context (Gill and Raine, 2014; Gill et al., 2012; Mommaerts et al., 2010; Rundlöf et al., 2015; Sandrock et al., 2014a; Whitehorn et al., 2012). The *A. mellifera* $LD_{50}$ value of 11,42 µg/bee is lower than the previously reported 38,83 µg/bee or 14,6 µg/bee (EPPO, 1992; Iwasa et al., 2004), suggesting higher sensitivity than previously thought. These discrepancies lead us to question the reproducibility of $LD_{50}$ values, especially when there is consistently a low dose-dependent response to thiacloprid. In contrast to Iwasa et al. (2004), we did not find a dose-dependent response following standard methods to construct an $LD_{50}$ curve based on 48-hour mortality. Furthermore, there is no dose dependent response when considering mortality measured across the entire 5-day experiment

represented by the hazard ratios. Our $LD_{50}$ values are significant when considering them with the probit analysis as the chi square value reflects that the observed model fits the predicted one. But as we point out here, without a dose-dependent response, the $LD_{50}$ value to assess insecticide sensitivity may not be very accurate. Our lack of dose-dependency may have resulted from our using newly emerged bees instead of older bees. Newly emerged bees have less developed and hardened exoskeletons (Falcón et al., 2014), which may increase the amount of absorption through the exoskeleton and therefore bees in this part of their life-cycle may be particularly sensitive to insecticide exposure by contact. Based on our results the $LD_{50}$ value appears to be close and reproducible for *A. mellifera* but not for *B. terrestris* or *O. bicornis* as the latter two have vastly different $LD_{50}$ values, which are not reflected in their overall hazard ratios determined from the 5-day survival curve analysis.

Despite selecting five insecticide doses, which includes a large range around the previously reported $LD_{50}$ value for *A. mellifera,* to construct a dose-response curve, one could argue that we should have administered a larger range of insecticide concentrations to achieve a dose-dependent response. However, when measuring the bee size underneath the dissecting microscope, we noticed crystallization of the insecticide in the hair on the back of thorax of the three bee's species treated with the highest dose of the insecticide, suggesting that higher doses of thiacloprid are not absorbed into the bee and instead crystalize on the surface of the bee. This lack of absorption would possibly explain why we do not see a relatively higher mortality in bees treated with higher doses. Therefore, we speculate that administering higher concentrations of insecticides would not result in higher mortality; instead we found, although not significantly, lower mortality. In summary, our results suggest that it may not be practical to administer thiacloprid via contact exposure, at very high concentrations, in the laboratory setting.

In theory if lower concentrations were administered than used in this study, perhaps we would have observed significantly lower mortality, but we did not detect lower mortality in bees treated with $1/50^{th}$ of $LD_{50}$ value (2%), which is considered to be a dose to which honeybees could be potentially exposed in a natural context (Smodiš Škerl et al., 2009), or even with a sublethal dose of $1/100^{th}$ of the $LD_{50}$ value (1%) for *Apis mellifera* (Vidau et al., 2011). Therefore, unless the dose-response curve (Figure 4) accompanies the $LD_{50}$ value so that a dose dependent response can be confirmed, the $LD_{50}$ value can only serve as a rough benchmark for gauging insecticide toxicity. Due to the lack of slope or strong correlation between dose and mortality, slight differences in mortality can cause large differences in predicting the $LD_{50}$ value across bee species.

Our results suggest that other toxicology models such as the threshold model or the more common Hormetic Dose-Response Model would not be a better fit unless there is a dose-dependent response to the insecticide administered (Calabrese and Baldwin, 2003). Another valid explanation, however, is that no dose-dependence exists for thiacloprid on an individual level, which has been demonstrated for toxicity exposure in other species (Sheehan et al., 1999); even a minute quantity of insecticide exposure causes bee mortality. Therefore, defining a sublethal insecticide dose based on an $LD_{50}$ value may have to be reconsidered until a dose-response curve can be demonstrated.

Despite a higher surface area to volume ratio in smaller bees in which a higher proportion of insecticide would be absorbed per unit area of bee tissue (Schmidt-Nielsen, 1984), we did not find bee size within a species to be a significant predictor of mortality. With respect to sex, we found a trend for higher female versus male *O. bicornis* mortality. The higher female sensitivity to insecticides may explain a male biased production when exposed to thiamethoxam and clothianidin in a natural context as the production of females rely on more parental investment such as more foraging trips and bees stressed from pesticide exposure may not be able to afford this additional energetic cost to produce females (Sandrock et al., 2014a).

*Osmia bicornis,* and wild bees in particular, seem to be more sensitive to pesticides than *A. mellifera* in a natural context, which is attributed to the large variation in life-history traits found across bee species and the lack of social 'buffer' (Rundlöf et al., 2015; Sandrock et al., 2014a). However, according to our results size does not correspond to insecticide toxicity. Instead a possible explanation for the variation observed in mortality to contact exposure across bee species might be due to the varying capacity of detoxification (Iwasa et al., 2004). We speculate that variation in other phenotypic traits across bee species, such as thicker hair, thicker wax

cuticle, or a denser exoskeleton (Moussian, 2010), might act as a physical barrier preventing the absorption of insecticides when exposed by contact.

In our experimental design the fact that each bee was maintained individually per cage, one might predict that *O. bicornis*, being a solitary bee (Seidelmann, 2014), would have the highest survival as social isolation has been shown to stress *A. mellifera* (Jorand et al., 1989). But our results show similar survival of these two bee species, though both were significantly lower than *B. terrestris*. Therefore, our design does not appear to negatively affect social bees, suggesting that our results are comparable across bee species. Moreover, when using this experimental design, there is an additional benefit of eliminating a possible cage effect as we can insure that there is no accidental ingestion of the insecticide from social behaviors such as allogrooming, trophallaxis, or incidental contact with other insecticide treated individuals.

In summary, our findings support the idea that more rigorous testing and a more holistic approach to assess insecticide toxicity are needed (Decourtye et al., 2013; Desneux et al., 2007; Mommaerts et al., 2010; van der Sluijs et al., 2013). In addition, our findings also support the idea that toxicity of insecticides based on honeybees cannot be generalized to other bee species or provide realistic risk assessment for bee species in a more natural context (Rundlöf et al., 2015). Even for honeybees it has already been demonstrated that relying only on $LD_{50}$ values for risk assessment does not account for the detrimental long-term impacts that neonicotinoids have in a more natural context (Sandrock et al., 2014b). Uhl et al. (2018), for example, found that *Osmia bicornis* is less sensitive to pesticides in comparison to *A. mellifera*, but the $LD_{50}$ value for *O. bicornis* was calculated from a lab experiment and then compared to previously published $LD_{50}$ values of *A. mellifera*. Since our results shows $LD_{50}$ values can vary greatly if there is no dose dependent response to the pesticide, our calculated $LD_{50}$ values should be interpreted with caution. To make $LD_{50}$ values more informative, error estimations of the dose-response curve slope and ideally the dose-response curve itself should always accompany the $LD_{50}$ value, which has been lacking in previous reports of *A. mellifera* (EPPO, 1992; FERA, 2013). Moreover, age differences should be accounted for as we find no dose-dependent response when testing newly emerged bees. Therefore, policy makers should not only be advised to demand more rigorous testing of insecticides in terms of considering their long term and sub-lethal chronic effects in a natural context (EASAC, 2015), they should also reconsider the extent to which the $LD_{50}$ value evaluation system can be used to assess the relative toxicity of insecticides across different bee species, even if it is considered as a standard benchmark to quantify pesticide toxicity.

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