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*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1651877> since 2018-04-05T13:54:37Z

*Published version:*

DOI:10.1007/s13592-017-0506-6

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# UNIVERSITÀ DEGLI STUDI DI TORINO

***This is an author version of the contribution published on:***

*Questa è la versione dell'autore dell'opera:*

***Toxicity of thiametoxam on in vitro reared honey bee brood***

***Apidologie 48 (5) 2017 : 635–643***

**DOI : 10.1007/s13592-017-0506-6**

***The definitive version is available at:***

*La versione definitiva è disponibile alla URL:*

<https://doi.org/10.1007/s13592-017-0506-6>

## **Toxicity of thiametoxam on *in vitro* reared honey bee brood**

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Short title: Thiametoxam toxicity on brood

### **Abstract**

Pesticides are a possible cause of pollinator decline and honey bee colony losses experienced in several countries in recent years. In the past years, many north-west Italian beekeepers reported the presence of dead brood in field apiaries during neonicotinoid coated maize sowing; therefore a possible role of these insecticides was suspected. The objective of this study was to test this hypothesis. Laboratory repeated-dose toxicity tests on *in vitro* reared larvae were carried out using thiamethoxam. The repeated treatment median lethal concentration (LC<sub>50</sub>) and the median lethal dose (LD<sub>50</sub>) 14 and 19 days after grafting were calculated and resulted of the same order of magnitude of realistic brood exposure under a worst case scenario. Various sub lethal effects, like brownish larvae, duplication of the pupal integument, delay in development and deformed adults were also observed.

**Keywords:** *Apis mellifera* / artificial rearing / cumulative toxicity / neonicotinoids / sublethal doses / thiametoxam

### **1. INTRODUCTION**

An extensive pollinator decline (Gallai et al., 2009; Potts et al., 2010; Nieto et al., 2014) and heavy honey bee colony losses (Janke and Rosenkranz, 2009; Neumann and Carreck, 2010) have been experienced in several countries lately. In the effort to bring light on the origin of these phenomena, several factors have so far been investigated. Furthermore, new features like the colony collapse disorder (CCD) syndrome have been described (vanEngelsdorp et al., 2009), nevertheless not a single cause was definitively identified (Maini et al., 2010; Ratnieks and Carreck, 2010; Williams et al., 2010). Therefore, it is most likely that some multifactorial effect is involved and lately the cumulative action of several stressors on the honey bee's immune response has been proposed as a unifying model (Nazzi et al., 2012; Di Prisco et al., 2013).

Among the various factors suggested, pesticides are one of the most relevant and in-depth investigated causes of colony losses (Blacquière et al., 2012; Krupke et al., 2012; Lu et al., 2012; Sluijs et al., 2013; European Academies Science Advisory Council, 2015). In particular, neonicotinoid insecticides - especially when used in seed coating - were greatly debated since heavy honey bee mortalities were observed in Italy and other European countries during maize sowing (Greatti et al., 2003 and 2006; Forster, 2009; Pistorius et al., 2009; Chauzat, 2010; Geest, 2012). At last, these facts led in 2008 to the ban of the use of neonicotinoids and fipronil as coating active ingredients for maize and sunflower. As a result, a dramatic reduction in the poisoning incidents reported in Italy was observed (APENET, 2009). In the years before the ban, many north-west Italy beekeepers reported dead brood with symptoms reminding the European foul brood (EFB) in their hives during maize sowing, but an EFB diagnosis was not confirmed. After the neonicotinoid and fipronil use had been restricted, this syndrome nearly disappeared; therefore a possible role of these insecticides in causing such symptoms was suspected and the objective of this study is to test this hypothesis.

Pesticide risk assessment on honey bee brood is far more complex than on adult workers, especially when chronic or sub-lethal effects are involved, since the honey bee brood develops in the hives, carefully reared inside comb cells, and exposure to pesticides is mostly mediated by both forager and nurse adult workers. Due to such difficulties, several laboratory (Czoppelt 1991; Davis et al., 1988; Mussen et al., 2004; Aupinel et al., 2007a and b; Gregorc and Ellis, 2011; Hendriksma et al., 2011; Tavares et al., 2015), cage (Schur et al., 2003), and field procedures (Oomen et al., 1992; Everich et al., 2009) have been proposed for testing pesticide toxicity on honey bee brood and to assess experimental results. Presently a universal consensus on a specific methodology for testing pesticides on honey bee brood has not yet been achieved (Aupinel et al., 2009; European Food Safety Authority, 2013). Nonetheless, as a first step to detect any possible relationship between neonicotinoids and honey bee brood anomalies, we planned some laboratory toxicity tests on *in vitro* reared larvae hoping that the results could contribute to the debate. A repeated dose exposure test modified from Aupinel's test (Aupinel et al., 2007a and b) was developed and thiamethoxam was used as toxic agent, since it is extensively used on crops and its toxicity to honey bees is well known even though the few available data on larvae are controversial (European Food Safety Authority, 2013a).

## **2. MATERIALS AND METHODS**

### **2.1. Brood rearing**

First instar worker larvae were collected from three *A. m. ligustica* colonies. Computer assisted wing venation morphometric analysis (Meixner et al., 2013) was used to assess the honey bee subspecies.

The hives were periodically checked to exclude the presence of the most common honey bee diseases (Shimanuki and Knox, 2000).

The larvae were grafted from a single brood comb into the wells of a sterile 48-well tissue culture plate; different plates were used for each tested colony. After grafting, the brood was reared in a dark incubator at 34.5 ( $\pm 0.5$ ) °C for 21 days to allow adult emergence. Larvae were kept at 96% relative humidity in hermetic desiccators containing dishes filled with a  $K_2SO_4$  saturated solution and pupae were kept at 80% relative humidity in hermetic desiccators containing dishes filled with a NaCl saturated solution.

The Aupinel's (Aupinel et al., 2005) semi-artificial diet was adopted. Larvae were fed daily for six days putting a food drop onto the well's bottom; diet composition and amounts fed to larvae are shown in Table I. Fresh royal jelly produced in the DISAFA's apiary, yeast extract from Biokar diagnostics (Beauvais, France), and sugars from Serva Feinbiobiochemica (Heidelberg, Germany) were used.

## 2.2. Toxicity tests

A thiamethoxam commercial formulation available in Italy (Actara<sup>®</sup>: 25.0% pure thiamethoxam, hydro dispersible granules) was used. It was mixed with the larval food so that known amounts of thiamethoxam were ingested by the larvae. Since larvae were in contact with the artificial food, some contact action could not be completely excluded.

Preliminary tests were carried out to determine the concentration range between 100% mortality and a mortality level not significantly different from that of the untreated controls. On this basis, the following thiamethoxam concentrations were used to determine the  $LC_{50}$ : 20, 10, 5, 2, 1, 0.5, and 0.2 mg/L. Three replications – one 48-well tissue plate per replicate – were done for each tested concentration and the untreated control, taking care that larvae from a different colony were used for each replicate.

From day 1 to day 5 – day 0 being the day the larvae were grafted – 1  $\mu$ L of a thiamethoxam suspension in water was added to the food in each well with the treated larvae, and 1  $\mu$ L of water was added in the wells with the untreated control larvae. The thiamethoxam suspension concentrations were such as to obtain the desired test concentration after dispersion of the given 1  $\mu$ L drop in the larval food. Doses fed to larvae are shown in Table I.

Mortality and any morphological and/or developmental anomaly that could be considered as a sub-lethal effect were visually checked and recorded daily immediately before feeding. Larvae that died within 24 hours after grafting were removed from the experiments and all computations in order to exclude grafting effects.

Two toxicity endpoints were evaluated: (i) cumulative mortality at day 14 after grafting as an assessment of living pupae (individual not moulted from prepupa to pupa on day 14 after grafting were considered as dead in the pre-pupal phase); (ii) number of emerged adults (non-emerged individuals on day 21 after grafting were counted as dead during the pupal stage).

### 2.3. Statistical analysis

The larval survival was analyzed with the survival analysis module of the statistical package PAST 3.05 (Hammer et al., 2001). The Kaplan-Meier survival curves for the tested concentration and the untreated control were drawn and each concentration curve was compared with that of the untreated control by means of the log-rank test. The Kaplan-Meier curves and the log-rank tests were computed according to Kleinbaum and Klein (2005). The obtained P-values were corrected with the Holm-Bonferroni procedure (Holm, 1979) to provide a multicomparison correction.

The median lethal concentration ( $LC_{50}$ ) at day 14 and day 19 after grafting and the relative 95% confidence intervals were calculated by means of *probit* analysis; the procedure devised by Milani (1995) was adopted. Since in the tests each honey bee larva ingests a total of 150  $\mu$ L of food from day 1 to day 5 (Table I), the repeated treatment cumulative median lethal dose ( $LD_{50}$ ) was obtained from the relative  $LC_{50}$ . Moreover the  $LC_{50}$  at day 14 and day 19 after grafting were calculated from mortality data relative to each tested colony; subsequently the three pairwise  $LC_{50}$  ratios were calculated and their statistical significance determined, under the null hypothesis that they are identical, following the procedure given by Robertson and Preisler (1992) and the computational details adopted by Laurino et al. (2013).

The frequency of individuals showing sub-lethal effects below  $LC_{50}$  concentrations were compared with those observed in the untreated controls by means Pearson's  $\chi^2$  test of independence with d.f. = 1 and significance *p* values calculated with a Monte Carlo permutation test with 9999 replicates. The statistical package PAST 3.05 (Hammer et al., 2001) was used for these computations.

## 3. RESULTS

Larval mortality 24 hours after grafting was always very low (0-3 dead larvae/plate; average survival  $\pm$  standard deviation =  $99.2 \pm 1.64\%$ ). In the following days, mortality increased throughout the experiments, although feeding and, therefore, thiamethoxam administration had ceased five days after grafting to allow fully grown larvae to transform into prepupae.

Kaplan-Meier survival dynamic curves show a regular increase in mortality from the untreated controls to the highest tested thiamethoxam concentration (Fig. 1). All tested concentrations resulted

statistically highly different from the untreated controls ( $P < 0.01$ ) except the lowest one (0.2 mg/L) which was hardly significant ( $P < 0.05$ ) as a consequence of the Holm-Bonferroni correction (Table II).

LC<sub>50</sub> 14 days after grafting, when pupation was achieved by all surviving individuals, resulted of 1.53 mg/L of larval food, nearly the double of the value of 0.79 mg/L of larval food reached five days later after adult emergence (Table III). Food intake was substantially completed by all larvae reaching the prepupal phase and therefore an individual intake of 150 µL of food was assumed to compute the LD<sub>50</sub> values shown in Table 3 even if larvae that died in the first days did not evidently ingest so much food. The three LD<sub>50</sub> values calculated for each colony used in the tests were substantially similar and no statistically significant difference emerged from the pairwise LC<sub>50</sub> ratios.

Various anomalies and sub-lethal effects were observed in the reared brood (Fig. 2). In general, sub-lethal effects were most abundant at lower concentrations than at higher ones, as far as the number of dead larvae and pupae diminished. They were categorised as shown in Table IV. The number of brownish worker larvae treated with thiamethoxam concentrations below LC<sub>50</sub> was significantly higher than that of untreated controls; pupation time was highly significantly delayed in treated brood and significantly more deformed adults emerged from it in comparison with untreated controls. Moreover, three pupae still enclosed in the larval integument were also observed in the treated brood.

#### 4. DISCUSSION

The very low larval mortality observed 24 hours after grafting indicates that grafting effects were negligible throughout the experiment thanks to the implementation of sound beekeeping practices in the management of the colonies the larvae were taken from and the correct grafting technique.

Feeding the larvae for five days with a constant thiamethoxam concentration instead of feeding them with a single dose in an early development stage, as suggested in most recently proposed protocols for testing pesticides on brood (Aupinel et al., 2007a and b; Hendriksma et al., 2011), seems a more realistic simulation of what may happen in a honey bee colony growing in an intensive farming area. The adopted repeated-dose approach could anyhow be considered more as a chronic toxicity test than as an acute toxicity test; therefore the use of Kaplan-Meier survival dynamic curves to depict the mortality increase, and of log-rank tests to compare them appear fully justified. Since the untreated control mortality was higher than the optimal levels suggested in the literature (Aupinel et al., 2005 and 2009), the more restrictive value of  $\alpha = 0.01$  was preferred to the usual  $\alpha = 0.05$  to evidence statistical significance. Under such conditions, the lowest tested concentration could be regarded as the NOAEC for thiamethoxam on brood in the adopted experimental conditions.

LC<sub>50</sub> figures obtained by us are of an order of magnitude lower than those reported by Tavares et al. (2015) for Africanized honey bees. Different responses to toxic compounds have been evidenced between honey bee strains (Ladas, 1972; Mansour and Al-Jalil, 1985; Suchail et al., 2000 Laurino et al., 2013), but in this case the adopted experimental designs – single dose *vs* repeated doses – should account for the difference.

The use of LC<sub>50</sub> – and/or the derived LD<sub>50</sub> – values to express the results of a repeated-dose toxicity test could be somehow questionable, since they are more often derived from acute toxicity tests. In any case, they prove to be rather useful when comparing laboratory results with a realistic landscape-level exposure to pesticides. Honey bee larvae are exposed to pesticides chiefly through the food nurse bees give them since the amounts of pollen and honey given to them is very low. Nurse bees may eat contaminated pollen and nectar and thus produce larval food containing pesticides (Rortais et al., 2005). Therefore effective exposure levels are difficult to determine. To overcome these difficulties, the EFSA guidance document on the risk assessment of plant protection products on bees (European Food Safety Authority, 2013b) proposes various shortcut values (SV) to be used for the estimation of oral exposure via nectar and pollen consumption under different scenarios. Since the reported EFB-like symptoms had been observed in field apiaries during maize sowing, the SVs proposed by EFSA for the adjacent crop (4.4 µg/larva) and/or field margin (2.2 µg/larva) scenarios can be considered as the most appropriate and should be chosen among the various SVs relative to honey bee larvae. These SVs refer to an a.i. application rate of 100 mg/m<sup>2</sup>, while the maximum application rate allowed for maize seed dressing in Italy was 6.3 mg/m<sup>2</sup> only (European Food Safety Authority, 2013a); therefore the SVs should be reduced to 272 and 136 ng/larva respectively. In any case it should be noted that such SVs are to be considered as a worst case scenario. Since the LD<sub>50</sub> values obtained in the present study are lower than the suggested SVs it is likely that, a widespread use of dressed seeds could expose honey bee larvae to dangerous thiamethoxam concentrations. Therefore extensive brood mortality and colony weakening would likely occur.

Various sub-lethal effects of pesticides on honey bee brood have been reported in the literature (Desneux et al., 2007; Wu et al., 2011; Blacquièrre et al., 2012; Simon-Delso et al., 2014) and some of them, like the delay in pupation and in adult emergence were observed also during this research. On the contrary developmental troubles leading to the emergence of deformed adults and the extremely rare – and therefore not statistically significant – appearance of pupae that are still enclosed in the larval integument were not commonly observed and seem to be rather unlikely for neurotoxic insecticides like thiamethoxam.



## **ACKNOWLEDGEMENTS**

The authors wish to thank Dr Noa Simon-Delso for her helpful criticism on an earlier draft of the manuscript.

**Toxicité du thiamétoxame sur le couvain d'abeille élevé *in vitro***

*Apis mellifera*/ élevage artificiel/ toxicité cumulative/ néonicotinoïde/ dose sublétales

**Toxizität von Thiametoxam für *in vitro* aufgezogene Brut der Honigbiene**

*Apis mellifera* / künstliche Aufzucht / kumulative Toxizität / Neonikotinoide / sublethale Dosis

/ Thiametoxam

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## FIGURE CAPTIONS

Figure 1. Kaplan-Meier survival dynamic curves of honey bee worker brood treated with decreasing thiamethoxam concentrations and of the untreated controls.

Figure 2. Sub-lethal effects observed in honey bee brood rearing tests.

**Table I.** Daily food volumes provided to the larvae, composition of the diet according to their age, and doses of thiamethoxam fed to larvae for each tested concentration from day 1 to day 5 after grafting, day 0 being the grafting day.

Rearing day	day 0	day 1	day 2	day 3	day 4	day 5	total
larval food amount ( $\mu\text{L}$ )	10	10	20	30	40	50	160
diet composition:							
royal jelly (%)	50	50	50	50	50	50	-
yeast extract (%)	1	1	1.5	2	2	2	-
D-glucose (%)	6	6	7.5	9	9	9	-
D-fructose (%)	6	6	7.5	9	9	9	-
dry matter* (%)	29.55	29.55	33.05	36.55	36.55	36.55	-
Thiamethoxam dose							
(ng/larva):							
20 mg/L	-	200	400	600	800	1,000	3,000
10 mg/L	-	100	200	300	400	500	1,500
5 mg/L	-	50	100	150	200	250	750
2 mg/L	-	20	40	60	80	100	300
1 mg/L	-	10	20	30	40	50	150
0.5 mg/L	-	5	10	15	20	25	75
0.2 mg/L	-	2	4	6	8	10	30

\* calculated from Crane (1990) for royal jelly and manufacturer's specifications for the remaining ingredients.

**Table II.** Survival dynamics of *in vitro*-reared honey bee worker brood treated with decreasing thiamethoxam concentrations compared with those of the untreated controls.

thiamethoxam concentration comparisons	log rank $\chi^2$	Bonferroni-Holm corrected $\alpha$		p(same) value
20 mg/L vs control	164.91	0.05	0.01	$9.57 \cdot 10^{-38}$ **
10 mg/L vs control	79.45	0.025	0.005	$4.96 \cdot 10^{-19}$ **
5 mg/L vs control	120.28	0.0166	0.00333	$5.49 \cdot 10^{-28}$ **
2 mg/L vs control	64.45	0.0125	0.0025	$9.92 \cdot 10^{-16}$ **
1 mg/L vs control	43.23	0.01	0.002	$4.86 \cdot 10^{-11}$ **
0.5 mg/L vs control	11.60	0.0083	0.00166	0.00066 **
0.2 mg/L vs control	8.35	0.0072	0.00142	0.0039 *

\*\* highly significant at  $p = 0.01$ ; \* significant at  $p = 0.05$



**Table III.** Thiametoxam repeated treatment median lethal concentration ( $LC_{50}$ ) and median lethal dose ( $LD_{50}$ ) for *in vitro*-reared honey bee worker brood at day 14 and day 19 after grafting. In brackets  $LC_{50}$  and  $LD_{50}$  upper and lower limits at  $P = 0.95$ .

	day 14	day 19
$LC_{50}$ (mg/L)	1.53 (0.33 - 3.26)	0.79 (0.25 - 1.39)
$LD_{50}$ (ng/larva)	229 (50 - 489)	118 (37 - 209)

**Table IV.** Number of sub-lethal effects showed by *in vitro*-reared honey bee worker brood treated below LC<sub>50</sub> thiamethoxam concentrations (0.5 and 0.2 mg/L) and in the untreated controls. Number of normal individuals are reported for comparison purposes.

sub-lethal effect	larvae		$\chi^2$	Monte Carlo p
	treated	control		
colour of larvae:				
brownish	141	114	4.98	0.0281 *
pearly-white	146	172		
pupation time:				
delayed	40	27	10.11	0.0017 **
normal	26	52		
pupal integument:				
double	3	0	3.42	0.1091 n.s.
normal	66	77		
adult workers:				
deformed	36	62	8.18	0.0073 **
normal	21	11		

\*\* highly significant ( $p < 0.01$ ); \* significant; n.s. not significant at  $p = 0.05$

Figure 1. Kaplan-Meier survival dynamic curves of honey bee worker brood treated with decreasing thiamethoxam concentrations and of the untreated controls.

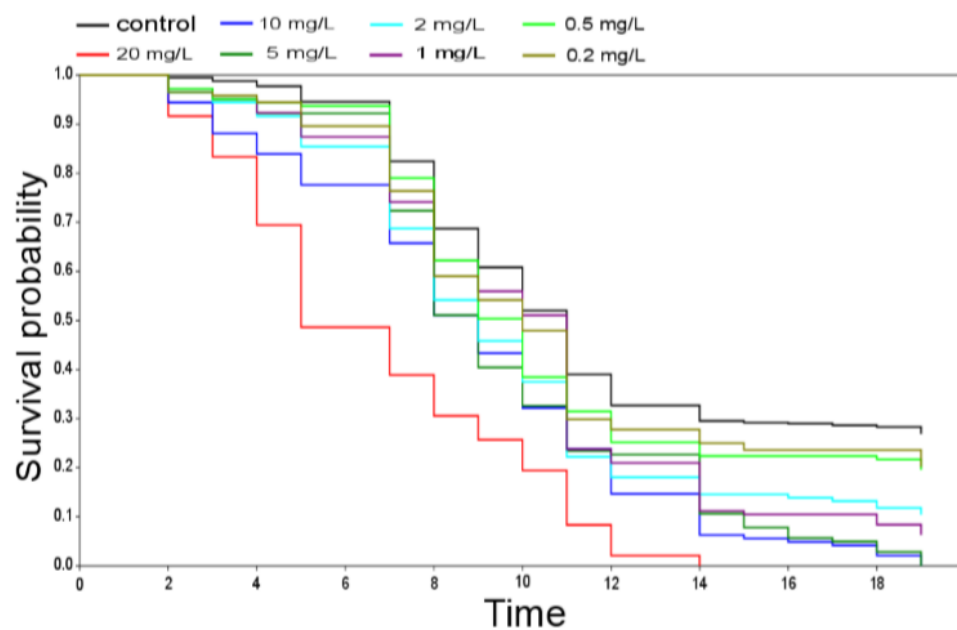
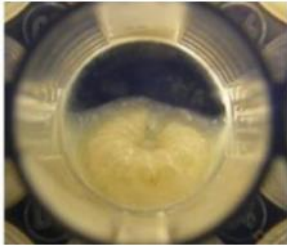
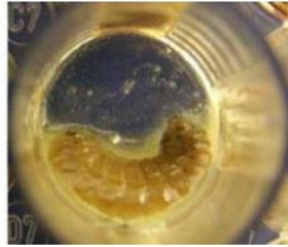


Figure 2. Sub-lethal effects observed in honey bee brood rearing tests.

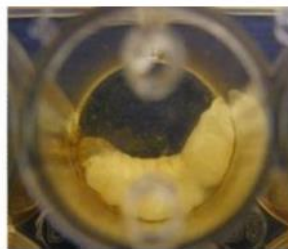
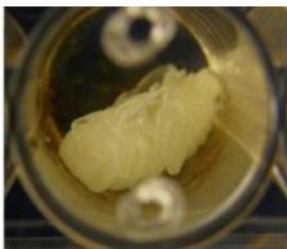
normal brood



abnormal brood



Colour of larvae



Pupal integument



Adults