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Toxicity of neonicotinoid insecticides on different honey bee genotypes

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Abstract

Toxicity effects of the neonicotinoid insecticides clothianidin, imidacloprid, and thiamethoxam were tested in the laboratory on different honey bee (*Apis mellifera* L.) genotypes belonging to the following subspecies: *Apis mellifera mellifera* L., *Apis mellifera ligustica* Spinola, and *Apis mellifera carnica* Pollmann. Oral and indirect contact trials were carried out on adult worker honey bees for each pesticide, using commercial formulations. The acute oral toxicity (AOT) LD_{50} and the acute indirect contact toxicity (ICT) LC_{50} were calculated. Mean AOT LD_{50} values at 24 hours (clothianidin 3.53 ng/honey bee; imidacloprid 118.74 ng/honey bee; thiamethoxam 4.40 ng/honey bee), 48 hours (clothianidin 3.35 ng/honey bee; imidacloprid 90.09 ng/honey bee; thiamethoxam 4.27 ng/honey bee), and 72 hours (clothianidin 3.28 ng/honey bee; imidacloprid 69.68 ng/honey bee; thiamethoxam 4.16 ng/honey bee) from test start were of the same order of magnitude of those reported in the literature for all three neonicotinoids. Statistically significant differences emerged in a few instances between groups of honey bees coming from the different hives tested for clothianidin, between the groups of honey bees coming from the single *A. m. mellifera* hive and the four *A. m. ligustica* hives tested for imidacloprid, and more extensively between the two *A. m. carnica*, the single *A. m. mellifera*, and the six *A. m. ligustica* groups of honey bees tested for thiamethoxam. ICT LC_{50} values were obtained for a reduced number of hives: the single *A. m. mellifera* and two *A. m. ligustica* hives for clothianidin, the single *A. m. mellifera* and one *A. m. ligustica* hive for imidacloprid, the single *A. m. mellifera*, three *A. m. ligustica* hives, and one *A. m. carnica* hive for thiamethoxam. Nevertheless statistically significant differences were observed for clothianidin and thiamethoxam, but not for imidacloprid. The results confirm that genetic differences in the response to pesticide toxic action exist in the honey bee, but they do not constitute the key factor involved in the uneven results observed in toxicity tests. In any case, the LD_{50} or other similar toxicity indexes should not be determined on a single colony.

Key words: *Apis mellifera*, acute oral toxicity, indirect contact toxicity, clothianidin, imidacloprid, thiamethoxam.

Introduction

Several plant protection products are dangerous for honey bees (*Apis mellifera* L.) and other pollinators in many ways (Riedl *et al.*, 2006; Desneux *et al.*, 2007). Therefore both active substances (a.s.) and formulated pesticides currently undergo various tests to assess the risk posed by them to honey bees, before their use in agriculture is allowed. For doing so, the European and Mediterranean Plant Protection Organization guidelines No. 170 (OEPP/EPPO, 2010a) and the relative risk assessment scheme (OEPP/EPPO, 2010b) are usually followed in the European Union. Such procedures substantially rely on Median Lethal Dose (LD_{50}) or other similar toxicity index determination in order to ascertain if risk levels associated with the tested a.s. are acceptable for honey bees. However, substantial differences often emerge when results of toxicity tests on honey bees performed by different laboratories are compared (Doucet-Personeni *et al.*, 2003; Aupinel *et al.*, 2009; Blacquière *et al.*, 2012; Simon-Delso *et al.*, 2012) and when different honey bee subspecies or even colonies of a single subspecies are tested in the same laboratory with the same methodology (Tahori *et al.*, 1969; Ladas, 1972; Mansour and Al-Jalil, 1985; Smirle and Winston, 1987; Suchail *et al.*, 2000). A different genetic response to toxicity tests is one of the possible explanations of such uneven results (Suchail *et al.*, 2001).

Genetic variability had been recognized in honey bees long ago (Rothenbuhler *et al.*, 1968; Rinderer, 1986), but a large amount of research has been devoted to subspecies identification and to the relative taxonomic implications (Ruttner, 1988; Engel, 1999), while functional characteristics, and especially those related to the response to toxic substances, have received less attention. Nevertheless it is well known that the subspecies differ not only in their morphology but also in several biochemical, physiological and behavioural traits and that similar differences can be observed also between ecotypes, populations, strains and even single colonies of the same subspecies (Ruttner, 1988). Therefore there is no reason why differences would not exist also in the response to toxic substances and some of them were experimentally evidenced in a few instances (Ladas, 1972; Mansour and Al-Jalil, 1985; Suchail *et al.*, 2000).

Beside genetic differences, the experimental procedures are often involved in the uneven honey bee response to pesticide toxicity, notwithstanding the efforts put into the devising of uniform guide-lines. Large differences in the temperature at which the tests are carried out, in the age of the honey bees used in the tests, and in the way honey bees are processed and dosed with the toxic substances can lead to substantially different results between different laboratories, even if the same guide line is followed (Ladas, 1972; Aupinel *et al.*, 2009; Medrzycki *et al.*, 2012). Also the very decision whether honey bees are dead or alive vary from considering them dead if they were unable to walk or fly (Iwasa *et al.*, 2004) to considering them alive until all physiologic activities are ceased.

A rather crude method has been often employed to determine whether the LD_{50} - or any other Median Effec-

tive Dose (ED_{50}) like the Median Lethal Concentration (LC_{50}) - of one group of tested animals significantly differs from that of another group or not: the 95% fiducial limits are calculated, checking whether or not the intervals resulting from these fiducial limits overlap (Robertson and Preisler, 1992). Such an approach provides very conservative results, while using 83% level for fiducial limits will approximate a 0.05 test (Payton *et al.*, 2003). In any case, great caution should be exercised when the results of an experiment are displayed with confidence or standard error intervals: whether or not these intervals overlap does not imply the statistical significance of the parameters of interest. An ED_{50} ratio test should be preferred to test effective doses, since it is a more powerful and statistically sound method of comparison (Robertson and Preisler, 1992; Payton *et al.*, 2003).

In recent years, neonicotinoid insecticides were involved in alarming bee mortalities in many countries (Greatti *et al.*, 2003 and 2006; Colin *et al.*, 2004; Janke and Rosenkrantz, 2009; Pistorius *et al.*, 2009; Forster, 2009; Marzaro *et al.*, 2011). They were also identified, along with other factors such as new and re-emerging pathogens, habitat loss, pests, and nutritional stress, as a potential contributing factor to CCD and as one of the environmental stressors contributing to pollinator declines (Byrne and Fitzpatrick, 2009; Gallai *et al.*, 2009; Decourtye and Devillers, 2010; Kamel, 2010; Maini *et al.*, 2010; Lu *et al.*, 2012). Several neonicotinoids, in fact, show a very strong toxicity to pollinating insects and in particular to the honey bee, causing also other effects such as behavioural disturbances, orientation difficulties, and social activity impairment (e.g. Guez *et al.*, 2001; Bortolotti *et al.*, 2003; Medrzycki *et al.*, 2003; Decourtye *et al.*, 2004a and 2004b; Ramirez-Romero *et al.*, 2005; Desneux *et al.*, 2007; El Hassani *et al.*, 2008; Yang *et al.*, 2008; Maini *et al.*, 2010; Matsumoto, 2013).

Recent investigations carried out by our laboratory on neonicotinoids by testing their toxicity on groups of honey bees coming from the different hives led to non uniform results. In these studies acute oral toxicity (AOT) was investigated on three different *Apis mellifera ligustica* Spinola strains (Laurino *et al*., 2010) and both AOT and indirect contact toxicity (ICT) were determined on another single colony of the same subspecies (Laurino *et al*., 2011). Therefore, it seemed appropriate to extend such investigations to other *A. mellifera* subspecies (*Apis mellifera mellifera* L. and *Apis mellifera carnica* Pollmann) and more colonies, using uniform procedures so as to highlight possible differences in sensitivity to clothianidin, imidacloprid, and thiamethoxam - the most widely used and toxic to honey bee neonicotinoids - that could be attributed to an uneven genetic response of the tested colonies.

Materials and methods

Honey bees

The results of the test carried out on four *A. m. ligustica* colonies had been published in previous papers (Laurino *et al*., 2010 and 2011), but the experimental data were assembled and uniformly analyzed with those obtained from five more colonies used for the present contribution. On the whole, tests were carried out on honey bees taken from nine hives (table 1); lig1, lig2, lig5, and lig6 were the colonies used in the previous investigations (Laurino *et al*., 2010 and 2011) and 42% of overall data discussed in the present contribution comes from them.

All the tested hives were periodically checked to exclude the presence of the most common honey bee diseases.

Morphometric analysis (Bouga *et al*., 2011) was used to assess the honey bee subspecies used in the trials.

Pesticides

Commercial formulations available in Italy were used. They contained: clothianidin (Dantop®: 50.0% pure a.s., hydro dispersible granules); imidacloprid (Confidor 200 SL®: 17.8% pure a.s., concentrated liquid soluble in water); thiamethoxam (Actara®: 25.0% pure a.s., hydro dispersible granules).

Table 1. Honey bee hives used in the tests and number of adult workers tested for each of the tested a.s. concentrations and for the untreated controls in each replication (number of replications in brackets) carried out determine acute oral toxicity (AOT) LD_{50} and indirect contact toxicity (ITC) LC_{50} of clothianidin, imidacloprid, and thiamethoxam. Strain A is the strain kept at the experimental apiary of the University of Turin; strains B to E come from different commercial queen breeders. Hive car1 was tested twice; test on car1b and car2 were performed in August and September, all other tests in June-July.

Hive	Subspecies	Geographic origin	Strain	Clothianidin		Imidacloprid		Thiamethoxam	
				AOT	ICT	AOT	ICT	AOT	ICT
lig1	*A.m. ligustica*	Piedmont (Italy)	A	30(2)	30(2)	-	-	30(2)	30(2)
lig2	"	"	A	20(2)	-	20(2)	-	20(2)	-
lig3	"	"	A	20(2)	20(2)	30(2)	20(2)	30(2)	20(2)
lig4	"	"	A	-	-	-	-	30(3)	30(3)
lig5	"	"	B	20(2)	-	20(2)	-	20(2)	-
lig6	"	"	C	20(2)	-	20(2)	-	20(2)	-
mel1	*A. m. mellifera*	South-East France	D	20(2)	20(2)	30(2)	20(2)	30(2)	20(2)
car1a	*A. m. carnica*	Croatia	E	-	-	-	-	30(2)	30(3)
car1b	"	"	E	-	-	-	-	30(2)	-
car2	"	"	E	-	-	-	-	30(2)	-

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 a.s. concentrations were used to determine the LC_{50}. AOT: clothianidin - 1.5, 0.75, 0.375, 0.15, 0.075, 0.0375, and 0.015 ppm; imidacloprid - 15, 7.5, 3, 1.5, 0.75, 0.3, and 0.15 ppm; thiamethoxam - 2, 1, 0.5, 0.2, 0.1, 0.05, and 0.02 ppm; ICT: clothianidin - 15, 7.5, 3.75, and 1.5 ppm; imidacloprid - 150, 75, 30, and 15 ppm; thiamethoxam - 20, 10, 5, and 2 ppm.

Test procedures

All the tests were carried out in the months of June and July except two thiamethoxam AOT tests on *A. m. carnica* honey bees (car1b and car2) which were performed in August and September.

AOT and ICT were assessed by means of the methods described in previous papers (Laurino *et al.*, 2010; 2011). Tests were performed in a dark room at 28-30 °C and 70% relative humidity. Foraging honey bees were taken from the flight board of the tested hive. Ten foraging honey bees were placed in each cage not later than 15 minutes from capture in order to minimize stress. Two or three cages were used for each replication and the tests were replicated two or three times (table 1). Replications with control mortality above 10% were discarded. The honey bees were considered as dead when they remained totally motionless during a 10 second observation period after having been lightly prodded with a fine paintbrush.

In AOT tests, the honey bees were administered a 25% sucrose solution, pure for untreated controls and in the other cases known amounts of the compounds to be tested were added. Solutions were made available to the honey bees for one hour; then honey bees could feed on sugar candy throughout the remaining part of the trial.

For ICT tests, chestnut (*Castanea sativa*) leaves were sprayed to drip with pure water, for untreated controls, or with water suspensions of the products to be tested and left to dry in the shade for at least three hours. The honey bees were allowed to walk freely on the cage bottom covered with leaves for three hours; then the leaves were removed. Throughout the trial, the honey bees were fed sugar candy.

Statistical analyses

The LC_{50} at 24, 48, and 72 hours from test start and the relative upper and lower 83% and 95% confidence intervals were calculated both for AOT and ICT tests by means of *probit* analysis; the procedure devised by Milani (1995) was adopted. Since in AOT tests each honey bee ingests on the average 35 µL of sucrose syrup during the allowed feeding period (Laurino *et al.*, 2010), the ingestion LD_{50} was obtained from the relative LC_{50}. LD_{50} could not be calculated for the ICT tests because the absorbed amount of the various a.i. cannot be determined. All the pairwise AOT LD_{50} and ICT 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); for each pairwise comparison the null hypothesis was checked at three probability levels ($P < 0.05$, $P < 0.01$, and $P < 0.001$) adopting 2.0 σ, 2.9 σ, and 3.4 σ respectively in the computations. As a safeguard against the risk deriving from performing multiple tests of statistical significance on the same data, the Bonferroni correction was adopted and only the AOT LD_{50} and ICT LC_{50} ratios for which the probability of the null hypothesis remained $P < 0.05$ after the correction were considered significantly different.

Results

Acute oral toxicity

Clothianidin LD_{50} values (figure 1) were rather uniform in the tested colonies, also when comparing results at 24, 48, and 72 hours; on the contrary, confidence intervals were narrower for some colonies and wider for others, especially lig1 and mel1, which are also the colonies with the lowest and highest LC_{50}, respectively. Therefore LC_{50} ratios are not statistically significant in most cases.

Honey bee mortality in response to imidacloprid was markedly irregular, especially at 24 hours (figure 2). Nevertheless the *A. m. ligustica* colonies that were used for the tests yielded comparable LD_{50} values, while mel1 results were so erratic that LD_{50} at 24 hours and the relative confidence intervals could not be calculated (figure 3). Besides mel1 LD_{50} values at 48 and 72 hours were significantly higher than those of lig2 to lig5 although the relative confidence intervals were exceedingly wide. Some statistically significant differences were also highlighted between *A. m. ligustica* colonies.

Thiamethoxam alone was tested also on *A. m. carnica* and both in early and late summer; remarkable differences in the results were obtained in the two periods, but not between the two colonies in the same period (figure 4). Both *A. m. carnica* colonies yielded LD_{50} values significantly higher than those of mel1 and of most of *A. m. ligustica* colonies.

Indirect contact toxicity

Clothianidin LC_{50} values (figure 5) were rather uniform in the tested colonies, but 95% confidence intervals were wider for mel1 than for the two tested *A. m. ligustica* colonies. Statistically significant differences emerged at 72 hours only.

Imidacloprid was tested in two colonies only and in both cases the LC_{50} upper 95% confidence interval at 24 hours could not be calculated; moreover, also the confidence intervals at 48 hours were extremely wide (figure 6). No statistically significant differences in LC_{50} ratios were observed for imidacloprid.

Also thiamethoxam LC_{50} values were rather uneven in the tested colonies (figure 7), but several statistically significant differences emerged, mainly at 72 hours.

Discussion and conclusions

Clothianidin and thiamethoxam AOT LD_{50} values were quite similar to and in the same order of magnitude of the data reported in the literature (Tomlin, 2003;

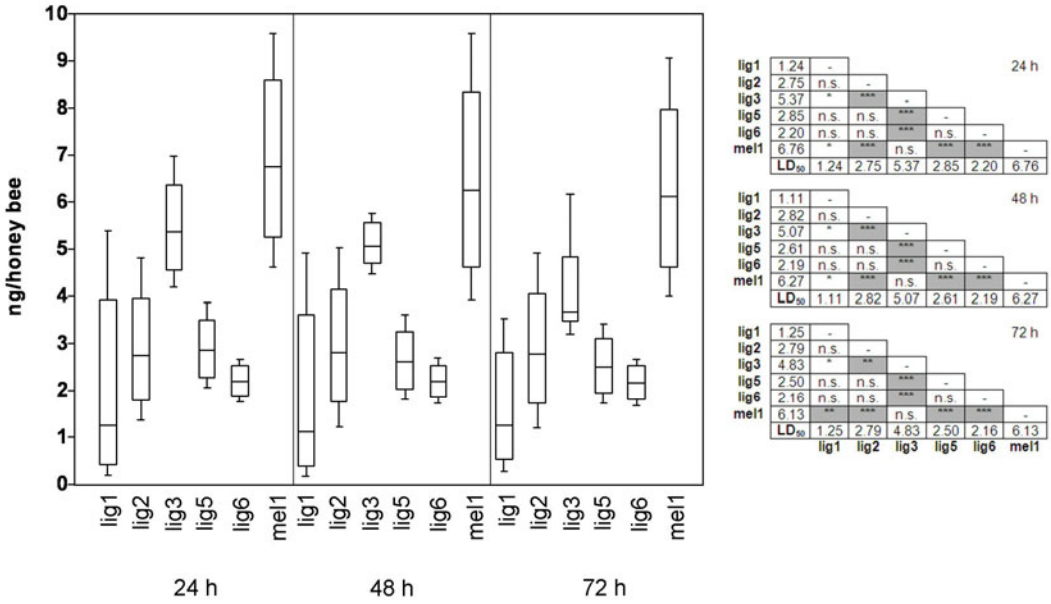

Figure 1. Clothianidin acute oral toxicity LD_{50} at 24, 48, and 72 hours with the relative upper and lower 83% (boxes) and 95% confidence intervals (vertical bars) and statistical significance of pairwise LD_{50} ratios for five *A. m. ligustica* (lig1, lig2, lig3, lig5, and lig6) and one *A. m. mellifera* (mel1) colonies. LD_{50} in ng/honey bee; n.s.: not significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; pairwise ratios evidenced in grey are significant at $P < 0.05$

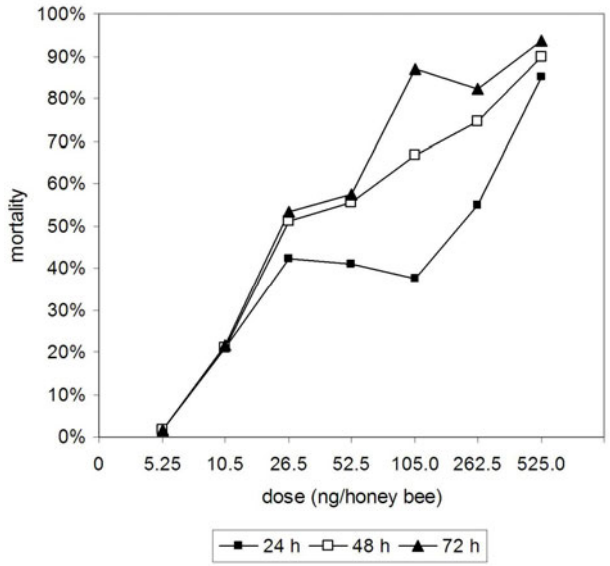

Figure 2. Bee mortality observed at 24, 48, and 72 hours after oral application of different imidacloprid doses. Data represent the means of four *A. m. ligustica* (lig2, lig3, lig5, and lig6) colonies.

Decourtye and Devillers, 2010); nevertheless statistically significant differences in LD_{50} were evidenced between several tested hives as a result of a till now unreported honey bee variability in AOT response to these neonicotinoids. Since imidacloprid has been in use for much more time than other neonicotinoids, its toxicity on honey bees has been extensively investigated and the several available LD_{50} determinations were critically collated and discussed by Doucet-Personeni *et al.* (2003). Our AOT LD_{50} values calculated for the tested *A. m. ligustica* colonies at 48 hours fell within the range they reported; moreover LD_{50} values calculated at 48 and 72 hours were comparable with the 37 ± 10 and 57 ± 28 ng/honey bee, respectively, obtained by Suchail *et al.* (2001) on *A. m. mellifera*. On the contrary, those relative to the single *A. m. mellifera* colony tested in the present study were substantially higher, but still within the extremely large range reported by Suchail *et al.* (2001), and similar to the 152.2 ng/honey bee indicated by Ruizhong *et al.* (1999). These contrasting results were likely due to the particular features of imidacloprid toxicology and its non-sigmoidal dose-effect relationships (Suchail *et al.*, 2000), which has been observed also in our tests. Surely methodological shortcomings of the oral toxicity bioassay, like the ingestion of unequal doses or the nutritive status of the honey bees at the time of application (Nauen *et al.*, 2001) could be taken into consideration (Schmuck *et al.*, 2003), but they should have occurred also with the other neonicotinoids.

Clothianidin and thiamethoxam LC_{50} values were rather similar also in the ICT tests, at least for the tested colonies, and markedly lower than those obtained for imidacloprid; in any case, they were less variable than AOT test results. A comparable difference between clothianidin and imidacloprid LC_{50} values was evidenced by Bailey *et al.* (2005) in their direct contact toxicity tests. Despite the different methodological approach, also acute topic contact toxicity shows a similar trend, with imidacloprid LD_{50} values on the average higher and more variable than those of clothianidin and thiamethoxam (Doucet-Personeni *et al.*, 2003; Iwasa *et al.*, 2004; Decourtye and Devillers, 2010). Recently, ICT tests carried out by Sgolastra *et al.* (2012) on clothianidin showed toxicity levels that are substantially lower than those reported in the present paper, but several practical differences in the test set out, alongside a higher genetic susceptibility to the a.i., can explain such results.

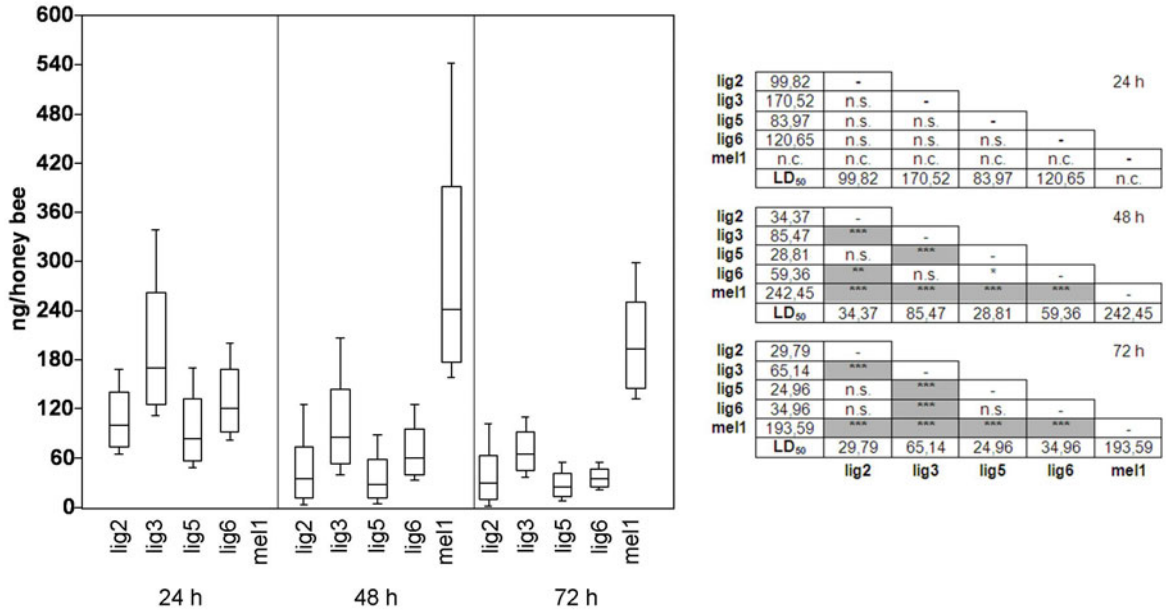

Figure 3. Imidacloprid acute oral toxicity LD_{50} at 24, 48, and 72 hours with the relative upper and lower 83% (boxes) and 95% confidence intervals (vertical bars) and statistical significance of pairwise LD_{50} ratios for four *A. m. ligustica* (lig2, lig3, lig5, and lig6) and one *A. m. mellifera* (mel1) colonies. LD_{50} in ng/honey bee; n.c.: not calculated; n.s.: not significant (P > 0.05); * P < 0.05; ** P < 0.01; *** P < 0.001; pairwise ratios evidenced in grey are significant at P < 0.05 after Bonferroni correction for multiple comparisons.

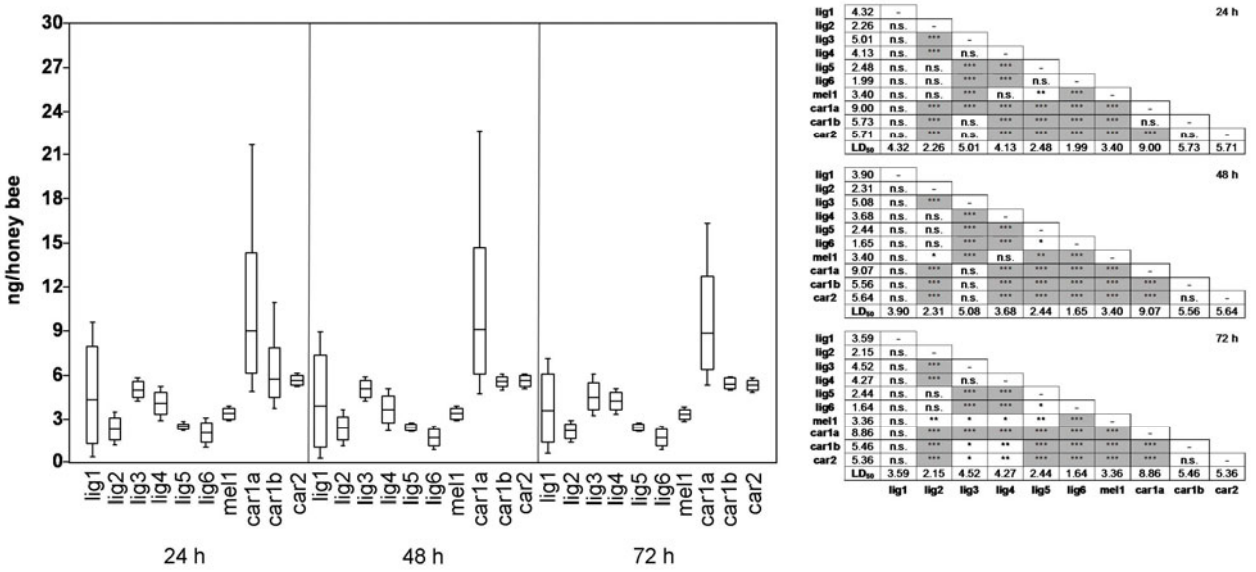

Figure 4. Thiamethoxam acute oral toxicity LD_{50} at 24, 48, and 72 hours with the relative upper and lower 83% (boxes) and 95% confidence intervals (vertical bars) and statistical significance of pairwise LD_{50} ratios for six *A. m. ligustica* (lig1 to lig6), one *A. m. mellifera* (mel1), and two *A. m. carnica* (car1 and car2) colonies. LD_{50} in ng/honey bee; n.s.: not significant (P > 0.05); * P < 0.05; ** P < 0.01; *** P < 0.001; pairwise ratios evidenced in grey are significant at P < 0.05 after Bonferroni correction for multiple comparisons.

Both AOT LD_{50} and ICT LC_{50} values tended to decrease with time in most cases, as it was to be expected since more bees die while time passes by; when values at 72 hours are higher than those at 24 and 48 hours, this is due to an increase in control mortality. Differences between 24, 48, and 72 hours were usually narrow, since mortality occurred mostly in the first 24 hours; only imidacloprid behaved in a somehow different way. In general, the most reliable results are obtained when the acute toxicity tests are carried out for 48 hours.

The AOT LD_{50} values obtained for the *A. m. carnica* hives tested in June-July and August-September, as limited as they could be, suggest that the time of the year in which tests are carried out is not an invariant parameter. Colony development and worker honey bee physiological conditions change during the season (Winston, 1987); in

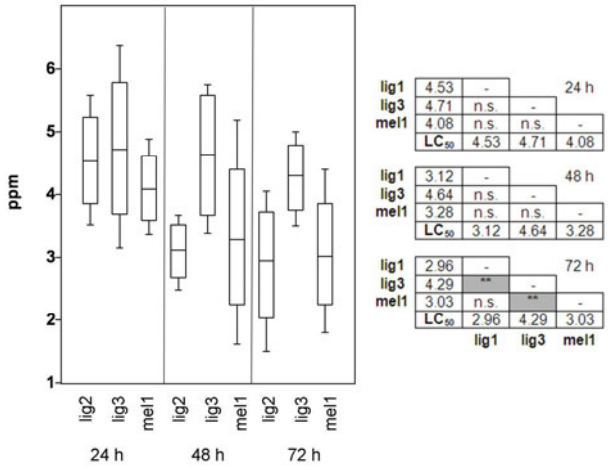

Figure 5. Clothianidin indirect contact toxicity LC_{50} at 24, 48, and 72 hours with the relative upper and lower 83% (boxes) and 95% confidence intervals (vertical bars) and statistical significance of pairwise LC_{50} ratios for two *A. m. ligustica* (lig1 and lig3) and one *A. m. mellifera* (mel1) colonies. LC_{50} in ppm; n.s.: not significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; pairwise ratios evidenced in grey are significant at $P < 0.05$ after Bonferroni correction for multiple comparisons.

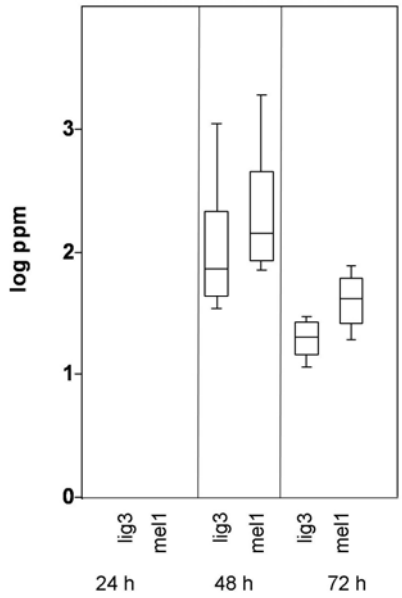

Figure 6. Imidacloprid indirect contact toxicity LC_{50} at 48 and 72 hours with the relative upper and lower 83% (boxes) and 95% confidence intervals (vertical bars) for one *A. m. ligustica* (lig3) and one *A. m. mellifera* (mel1) colonies.

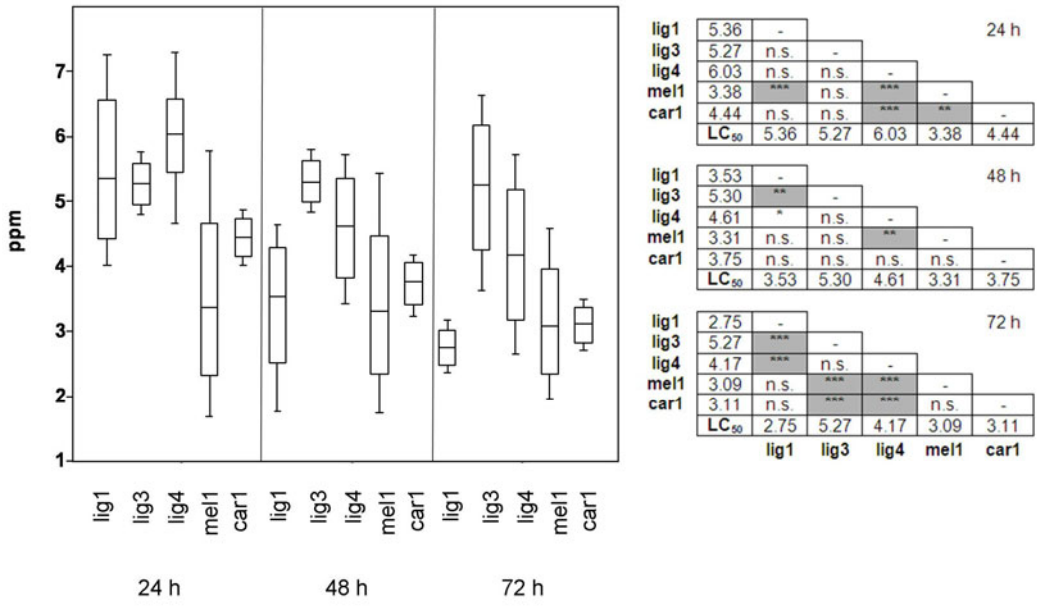

Figure 7. Thiamethoxam indirect contact toxicity LC_{50} at 24, 48, and 72 hours 72 with the relative upper and lower 83% (boxes) and 95% confidence intervals (vertical bars) and statistical significance of pairwise LC_{50} ratios for three *A. m. ligustica* (lig1, lig3, and lig4), one *A. m. mellifera* (mel1), and one *A. m. carnica* (car1) colonies. LC_{50} in ppm; n.s.: not significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; pairwise ratios evidenced in grey are significant at $P < 0.05$ after Bonferroni correction for multiple comparisons.

particular, under the climatic conditions of the area where the tests were carried out, winter honey bees were still present in August-September. Therefore, the period of the year during which toxicity tests are carried out should be clearly indicated when reporting the results and taken into consideration when comparing experimental data coming from different laboratories.

The results confirm that genetic differences in response to neonicotinoid toxic action exist in the honey bee, as firstly shown by Suchail *et al.* (2000), but no evident trend can be highlighted either in relation to subspecies or between AOT and ICT tests; additionally, differences were evidenced also between *A. m. ligustica* colonies. Therefore, in order to obtain reliable figures,

the LD_{50} or other similar toxicity indexes should be determined on at least three unrelated colonies and not on a single one as currently done. In any case, genetic differences do not constitute the key factor involved in the uneven honey bee response to pesticide toxicity tests carried out by different laboratories, and other disturbances, probably due to uneven experimental procedures, should be taken into account.

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