*Bulletin of Insectology* ***70*** *(1): 83-89, 2017*
ISSN 1721-8861

# Toxicity assessment on honey bee larvae of a repeated exposition of a systemic fungicide, boscalid

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## Abstract

Bees are key participators to the fertility of plants and yet they are suffering from losses and disorders. In other studies, double the amount of fungicides were found in colonies showing disorders than in healthy ones, with boscalid among the most frequently detected residues. Boscalid was mainly found in bee bread, main ingredient of larvae food and consequently exposing larvae to the compound in the long run. Here, we wanted to understand if boscalid could be toxic for immature stages of honey bees. Honey bee larvae were administered food to a range of doses from 0.04 to 40.25 µg boscalid/larva over 3 days. The NOED and LD50 at D8 were 4.025 µg a.i./larva and 86.786 µg a.i./larva, respectively; at D15 were 40.25 µg a.i./larva and 78.782 µg/larva, respectively; and at D22 were 40.25 µg/larva and 75.191 µg/larva, respectively. Worst-case field observed doses are 26 ppm in pollen and 1.43 ppm in nectar. Calculated concentration of boscalid safety for larvae would be 741 ppm for pollen and 27 ppm for nectar. However, our results with the active ingredient could be different than those observed for queen larvae, those obtained with the formulated product containing boscalid or in the field considering the exposure of bee hives to multiple pesticide contaminants.

**Key words:** honey bees, larvae, boscalid, fungicide, toxicity assessment, repeated exposure.

## Introduction

The honey bee *Apis mellifera* L. is a bee species present worldwide, serving humans and nature with regulating ecosystem services like pollination, but also providing food and pharmaceutical products and many other socio-economic ecosystem services (Millennium Ecosystem Assessment, 2005; Kremen *et al*., 2007; van der Sluijs *et al*., 2013). Unfortunately a wide spread phenomenon of increasing honey bee colony losses and disorders has been settled in most of the countries were the subject has been a matter of study (van der Zee *et al*., 2012; 2015; Yamada *et al*., 2012; Lu *et al*., 2014; Pistorius *et al*., 2015; Tosi *et al*., 2016). These trends are part of the general framework of pollinators decline described in Europe and other countries for several years now (Biesmeijer *et al*., 2006; Kosior *et al*., 2007; Goulson *et al*., 2008, Maini *et al*., 2010; Bommarco *et al*., 2011; Van Dyck *et al*., 2009; Bartomeus *et al*., 2013; Carvalheiro *et al*., 2013; Nieto *et al*., 2014, Renzi *et al*., 2016). The current situation threatens the continuity of pollinators role and the balance in nature (Garibaldi *et al*., 2014, IPBES, 2016). Possible stressing factors proposed to explain such trends are pathogens, habitat loss, pesticide exposure, nutrition (or lack of it) and climate change, and in the case of managed bees, management practices, all of them intervening with different relative weight depending on the context (Goulson *et al*., 2015).

In the southern part of Belgium, a field case study was carried out by Simon-Delso *et al*. (2014) in 2011-2012 aiming to clarify the phenomenon of unexplained winter colony losses beekeepers were reporting since the 2000s. Most of the mentioned stressors were included in the study. They found that honey bee colony disorders during the winter were strongly correlated with the presence of fungicides in the beekeeping matrices collected in July and September and with the area of arable crops around the apiary. The main pesticide residue detected in that study was boscalid, a systemic, fat-soluble and persistent carboxamide fungicide (Fungicide Resistance Action Committee -FRAC- Group 7) authorized to be used in Belgium in many crops attractive or not for bees, i.e. cereals, potatoes, oilseed rape, fruit trees, berries, aromatic and ornamental plants, vegetables, hops, vine plants (Phytoweb, 2015). Boscalid is a pesticide molecule widely used in agriculture and, as a result, widely present in beekeeping matrices. Numerous publications have found levels of contamination of beekeeping matrices like bees, pollen, bee bread, honey or wax, between some parts per billion (ppb, µg/kg) levels and up to 26.2 parts per million (ppm, mg/kg) (Aubee and Lieu, 2010; Johnson *et al*., 2010; Mullin *et al*., 2010; Wallner, 2011; Wu *et al*., 2011; Stoner and Eitzer, 2013; Simon-Delso *et al*., 2014), which corresponds to the largest amount found in pollen loads (Wallner, 2011). Nectar residues have been described up to 1.43 ppm (Wallner, 2011) and wax amounts ranged from 1 - 13 ppm (Aubee and Lieu, 2010; Ravoet *et al*., 2015, respectively). These concentrations can be translated into dose per larva basing on food consumption data (USEPA, 2014), the total amount of pollen, nectar and royal jelly ingested by a worked larva being 216.3 mg. This would translate into a nominal dose of 5.6 µg/larva should we assume that all these matrices were contaminated at the same level (26 ppm), or 1.19 µg/larva should we assume pollen and royal jelly residues being 26 ppm and nectar ones 1.43 ppm. This rea-

soning is conservative and the worst case might lead to even higher exposure levels. In fact, due to the lack of knowledge about the fate of this a.i. in the nurse bee body, it is unknown whether its concentration in royal jelly may be similar to the one found in pollen, may be lower or even higher. The latter possibility, if relevant, could be due either to accumulation mechanisms or to the fact that the protein/aminoacid content in pollen pellets (6.31-37.40% of dry matter, Todd and Bretherick, 1942; Somerville, 2001; Tasei and Aupinel, 2008; Forcone *et al.*, 2011; Liolios *et al.*, 2015; Taha, 2015) is lower than in the royal jelly (30-50% of dry matter, Scarselli *et al.*, 2005), even so if we consider that royal jelly is always contaminated by pollen grains. In other words, in order to produce 1 mg of royal jelly much more pollen is consumed and if all the a.i. contained in this pollen finished in the royal jelly, the relative concentration would be much higher.

Boscalid exhibits its mode of action through the inhibition of the mitochondrial respiration (succinate deshydrogenase) and subsequently reduction of adenosine triphosphate (ATP) in fungal cells (Stammler *et al.*, 2008). The authorization dossier of boscalid shows a low acute toxicity to honey bees, with an acute LD50 (both oral and contact) of >11 µg/bee (Aubee and Lieu, 2010) and >100 µg/bee (EC, 2008). For this reason, no higher tier studies were submitted by the producer, BASF AG, for authorization purposes. Preliminary tests done with boscalid did not induce to consider it as an Insect Growth Regulator (IGR). As a result, no complementary studies were carried out specifically targeting the toxicity of larvae and no toxicity problems were evaluated at colony level. However, recently boscalid was found to be linked to less pollen consumption and digestion in bees, lower ATP concentrations in the thoracic muscle tissue and higher virus titers (DeGrandi-Hoffmann *et al.*, 2015).

Following the results found in the field case study, we hypothesize that honey bees could be chronically exposed to boscalid residues from the first life stage. In this study, we aim to evaluate whether the larval toxicity of boscalid is a plausible explanation of the elevated colony mortality by using the new OECD methodology for larva toxicity of repeated exposure.

## Materials and methods

The methodology defined by the OECD Draft Guidance Document on Honey Bee (*Apis mellifera*) Larval Toxicity Test, Repeated Exposure (revision of April 2015) was used (OECD, 2015). A schematic representation of the most important steps of the larval repeated exposure test can be found in figure 1. Accordingly, the queen bees of three different queen-right healthy colonies were caged for around 30 hours, then released and three days later, the caged frames brought to the lab. A minimum of 144 first instar (L1) larvae were grafted per colony with the help of a paintbrush and deposited into crystal polystyrene grafting cells placed at sterile polystyrene 48-well tissue culture plates with flat bottom, treated by vacuum gas plasma (Falcon®), containing an uncontaminated diet. On the third day (D3) apparently dead larvae were removed and the living ones were randomly allocated to the different test plates ensuring that at least 12 larvae per colony were present in each plate. There were no deviations from the official OECD protocol. Temperature and relative humidity conditions during the test were monitored and are presented in supplemental material (table S1).

### Diets and test solutions

The diet composition as well as the test solutions administered and larvae used per treatment are specified in supplemental material table S2 and S3, respectively. Boscalid has a solubility in water of 4 mg/L and 180 g/L in acetone. To enable full solution, pure boscalid (Sigma-Aldrich, batch number SZBC180XV, 99.9% purity) was diluted into acetone and the test diet contamination was carried out into a cold chamber at 4 °C to reduce solvent evaporation. Dimethoate (Sigma-Aldrich, batch number SZBC243XV, 99.5% purity) was used as toxic standard and the respective diet was prepared as described above. Seven polystyrene plates were used, one for each treatment, namely one water control, one solvent control, 4 boscalid doses and the toxic standard.

Given that boscalid is supposed to be not toxic to bees, nor to be an IGR and because, to our knowledge, there are no previous studies carried out with this active ingredient on honey bee larvae, a range-finding test was

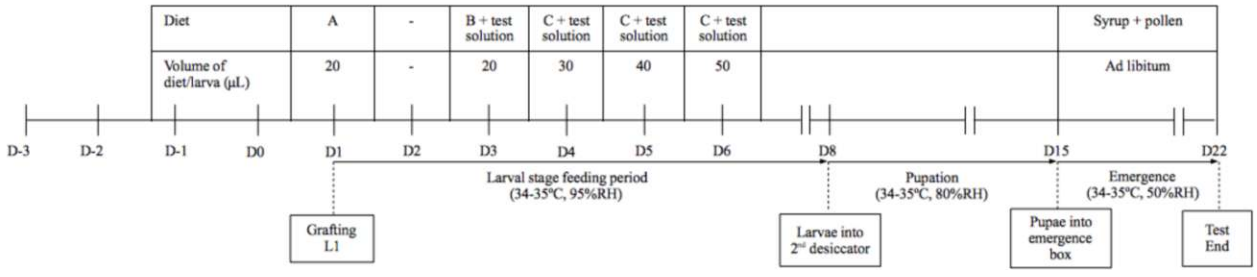


**Figure 1.** Schematic representation of the important steps of the larval repeated exposure toxicity test (D = day; RH = relative humidity). Adapted from OECD (2015).

**Table 1.** Toxicological endpoints and statistical analyses used in D8, D15 and D22.

| Assessment day | D8<br>µg a.i./larva | D15<br>µg a.i./larva | D22<br>µg a.i./larva |
|---|---|---|---|
| NOED | 4.025* | 40.25* | 4.025* |
| Test | $\chi^2$ 2 × 2 test with Bonferroni correction | Step-down Cochran.Armitage | Step-down Cochran.Armitage |
| $LD_{50}$ (range CL 95%) | 86.78 (65.75-114.55) | 78.78 (58.60-105.92) | 54.53 (36.72-80.97) |
| Test | Trimmed Spearman-Karber | Trimmed Spearman-Karber | Trimmed Spearman-Karber |

* Control and solvent control data are pooled.

used for the determination of NOED (No Observed Effect Dose) and LD50. Depending on the results, a subsequent finer test could be envisaged, but according to our results it was not necessary. A geometric ratio of 10 was used, starting at a high concentration of 57.5 mg/mL (261.4 mg a.i./kg diet) and reaching to 0.0575 mg/mL. Ten µL of each boscalid solution were added to the different diets. Dimethoate was dosed at 1.082 mg/mL (49.2 mg a.i./kg diet) in demineralized water and 100 µL were added to the diets (prepared in vials of 2 mL). Pesticide concentrations in the different diets and dose received per larvae can be found in supplemental material (table S3).

Both boscalid and dimethoate are described as stable and non-volatile (PPDB, 2015). For this reason, the diets were contaminated and homogenised at the beginning of the assay and kept in a cold chamber (4 °C) until the end of the test (22 days). Before administration respective diets were warmed to the temperature of the incubator (34.5 ± 0.5 °C).

### Effect observations

Individual survival was tested based on the development capacity of larvae or on the reaction of individual larvae to stimulus (gentle touch with a paintbrush). NOED determination was based on survival. Therefore a non-developed larva or a larva that did not react to stimulus were considered as dead. Results were registered and plates were photographed at days 4 (D4), 5 (D5), 6 (D6), 7 (D7), 8 (D8), 15 (D15) and 22 (D22) of the test (supplemental material, figure S1). On D8, the end of the exposure phase, it was recorded if larvae had consumed or not the diet provided, which was assessed following visual inspection. Non-consumed diet is presented as a thin layer of gel in the bottom of the cells. Cells were not weighted in order to establish food consumption as it would be difficult to discern the food weight and the larva one. On D22 the non-emerged adults were counted, recorded and photographed. The number of deformed bees was also recorded.

Plates were always processed from the control up to those containing the highest dose and toxic in order to avoid contamination. The paintbrush was thoroughly cleaned with water after each evaluation.

### Statistical analyses

The numbers of dead larvae (at D8), pupae (at D15) and non-emerged adults (at D22), in respect of the total amount of active ingredient (a.i.) consumed by the individuals, were processed using the software ToxRat Professional, version 3.2.1 - 2015.

Fisher's Exact Binomial Test was carried out in order to compare the mortality in water and solvent control.

The LD50 values and their confidence limits (at 95%), both expressed in µg a.i./larva, were estimated using the Spearman-Karber method (Carter, 1994) (table 1).

The NOED expressed in µg a.i./larva, was calculated with $\chi^2$ 2 × 2 test with sequential Holm-Bonferroni correction for D8 and with Step-down Cochran-Armitage test for D15 and D22 accordingly to the structure of data.

## Results

The difference in larval mortality in the control and control solvent (0.5% acetone) was not significant at D8 (two tailed Fisher's Exact Binomial Test, $p = 0.23$), D15 ($p = 1.00$), D22 ($p = 0.3$). The two control groups showed a cumulative larval mortality at D8 of 9% (n = 44) and 2.5% (n = 40), respectively (figure 2). This seems within the accepted range of control mortal toxicity previously observed in in-vitro larval rearing (Aupinel *et al*., 2007, Crailsheim *et al*., 2013). Thus, accordingly to OECD GD 54 (OECD, 2006), for those two assessment periods the data were pooled in a unique control group. The treatment response was corrected by the control response using Abbott's formula (Abbott, 1925).

At the key test dates of D8, D15 and D22, LD50 was 86.78 µg a.i./larva (CI: 65.75 - 114.55), 78.78 µg a.i./larva (CI: 58.60 - 105.92), 75.19 µg a.i./larva (CI: 54.99 - 102.82), respectively. NOED values were 4.025 µg a.i./larva for D8, and higher or equal to 40.25 µg a.i./larva for D15 and D22 (table 1). Raw data of absolute mortality in each treatment and at each assessment time are reported in table 2.

It must be stressed that during the experiment the 50% relative mortality was never reached in the boscalid treated plates. Thus the LD50 values are higher than the highest tested dose, being a product of mathematical extrapolation. This situation results in relatively high Confidence Limit range. Nevertheless it is possible to assert with an acceptable uncertainty level (5%) that the LD50 of boscalid is not higher than 115 µg a.i./larva but the toxic effects may be observed above 4 µg a.i./larva (NOED at D8).

On D22, 9 dead emerged bees out of 31 surviving larvae were found in the control, while in the other groups dead emerged bees ranged between 0 and 2. These results have no impact on the validity of the control

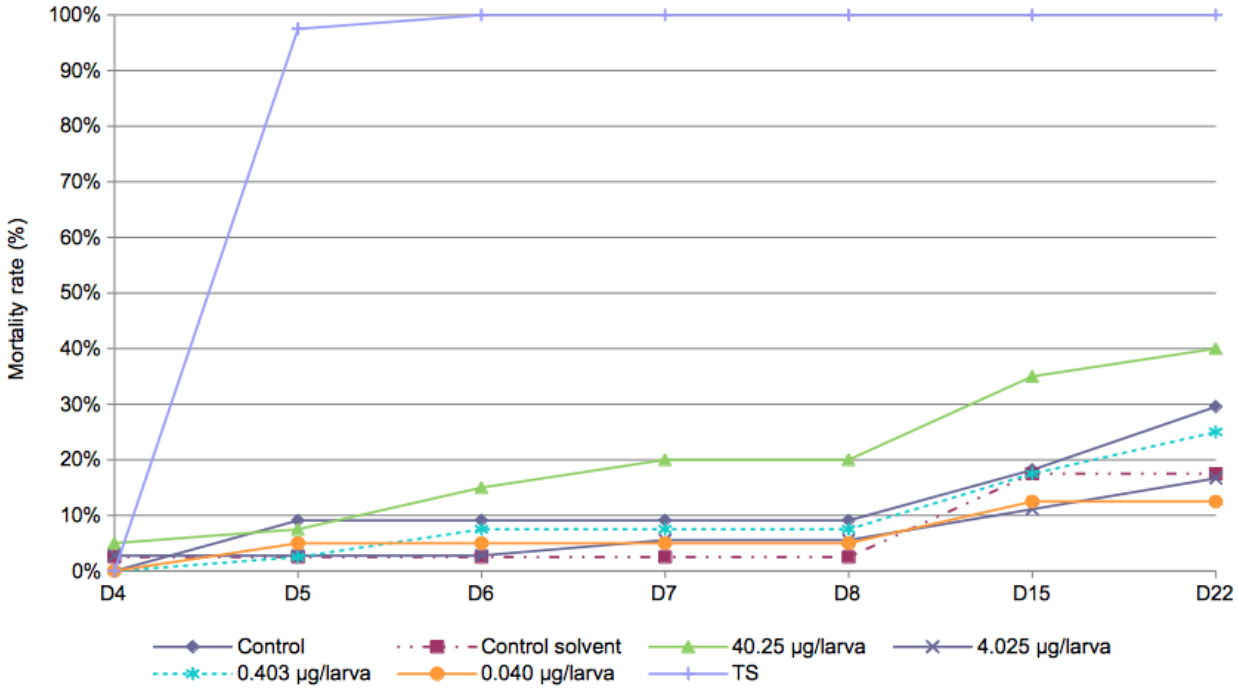


**Figure 2.** Evolution of mortality after an oral exposition of a diet alone (control), a diet with solvent (control solvent), a diet with four boscalid concentrations or one dimethoate concentration (TS) from day 4 (D4) to day 22 (D22).

**Table 2.** Cumulated mortality and effects observed in larvae during the 22 days of test.

| Treatments | Number of larvae at D3 | | D4 | D5 | D6 | D7 | D8 | D15 | D22 | Dead larvae % | Dead pupae % | Total mortality % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | 44 | Dead | 0 | 4 | 4 | 4 | 4 | 8 | 13 | 9.09 | 20.45 | 29.55 |
| | | Affected | 7 | 1 | 0 | 0 | 0 | 0 | 9 | | | |
| Control (acetone) | 40 | Dead | 1 | 1 | 1 | 1 | 1 | 7 | 7 | 2.50 | 15.00 | 17.50 |
| | | Affected | 8 | 0 | 0 | 0 | 0 | 0 | 1 | | | |
| Solution 1 (boscalid) | 40 | Dead | 2 | 3 | 6 | 8 | 8 | 14 | 16 | 20.00 | 20.00 | 40.00 |
| | | Affected | 6 | 2 | 4 | 0 | 0 | 0 | 2 | | | |
| Solution 2 (boscalid) | 36 | Dead | 1 | 1 | 1 | 2 | 2 | 4 | 6 | 5.56 | 11.11 | 16.67 |
| | | Affected | 6 | 0 | 0 | 0 | 0 | 0 | 1 | | | |
| Solution 3 (boscalid) | 40 | Dead | 0 | 1 | 3 | 3 | 3 | 7 | 10 | 7.50 | 15.50 | 25.00 |
| | | Affected | 12 | 0 | 0 | 0 | 0 | 0 | 0 | | | |
| Solution 4 (boscalid) | 40 | Dead | 0 | 2 | 2 | 2 | 2 | 5 | 5 | 5.00 | 7.50 | 12.50 |
| | | Affected | 5 | 0 | 0 | 0 | 0 | 0 | 1 | | | |
| Toxic standard (dimethoate) | 40 | Dead | 0 | 39 | 40 | 40 | 40 | 40 | 40 | 100.00 | 0.00 | 100.00 |
| | | Affected | 40 | 0 | 0 | 0 | 0 | 0 | 0 | | | |

according to OECD standards. Some deformed emerged bees (i.e. abnormal abdomen shape, humpbacks, n = 4) were observed in the boscalid 57.5 mg/mL group, while the number of deformed emerged bees in the other groups was between 0 and 2.

## Discussion

Larval mortality on D22 of the control water was just below the validity criteria accepted in tests carried out for regulatory purposes (30%) mentioned in OECD guidelines. However, the solvent control showed acceptable mortality rates and indicate that larvae used for the test were in good conditions. The source of a higher mortality in the water control could have been the fact that this plate was always processed first, possibly serving as a training plate. This could be the reason behind the larger number of dead emerged bees from the control plate. Operators observing recurrently these effects could foresee to include an extra training control water plate to be handled always at the beginning of the manipulations/observations.

The number of deformed emerged bees remained low and was mainly linked to abnormal abdomen shape (humpbacks). These malformations have been previ-

ously described and could be an artefact of the methodology used as described by Riessberger-Gallé *et al*., 2008. In real conditions, larvae are reared on vertical frames, while the plates in our test remained always horizontally. Riessberger-Gallé *et al*., 2008 proposed solutions to this issue.

Larvae mortality was observed to be 97.5 and 100% in the toxic standard (dimethoate) group after two and three days of exposure, respectively, all larvae seeming to have been affected following the first day of exposure. This may indicate an acute toxic dose of dimethoate. Aupinel *et al*., 2007 show a weak mortality dynamics at doses lower than 40 mg/kg dimethoate. It could be recommended to reduce the concentration of dimethoate in the toxic standard to mimic chronic toxicity dynamics.

The consumption of pollen and nectar during the larval development (5 days for worker larvae and 6.5 days for male ones) has been already estimated to: (1) 59.4 - 98.2 mg sugar in nectar for workers and male larvae, respectively (Rortais *et al*., 2005); and (2) 5.4 mg pollen for worker larvae (Babendreier *et al*., 2004). Based on these findings, the residues of boscalid found in beekeeping matrices and our results, the calculated concentration of boscalid safety for larvae would be 741 ppm for pollen and 27 ppm for nectar. Our results show that the active ingredient boscalid alone does not seem to be toxic to worker larval development and survival at the concentrations mentioned in literature for the beekeeping matrices. In particular, Simon-Delso *et al*., 2014 found boscalid residues in bee bread both morbid (n = 24) and healthy colonies (n = 10), with concentrations between 0.005 and 1.3 ppm in the former, while boscalid residues remained lower to 0.058 ppm in the latter. However, assuming that toxicity of boscalid on queen larvae is similar to that of worker bees and following our reasoning in terms of residue content in royal jelly, queen larvae would be exposed to doses just above the NOED (4.56 µg boscalid/larva). These estimations might be far from reality. This is why it would be worthwhile investing future research efforts in clarifying the amount of toxicants ending up in royal jelly.

The systemic properties and persistence of boscalid increases its potential to be found in nature over long periods of time. Indeed, pollen pellets collected by bees have proved to be contaminated with boscalid over periods of three months at least (personal observation). The presence of boscalid in bee bread samples collected in July-August indicates even previous exposure to this compound. This involves chronic exposure of several generations of bees within the colony and a plausible exposure of the queen to the same chemical over months. As a result, not only larvae could be exposed over their complete developmental period, but also after emergence and during their life as in-hive bees. Future laboratory experiences could envisage to evaluate the impact of a life time exposure, including larval development, on bees. This would be specifically interesting for compounds with similar physicochemical characteristics as boscalid.

However, our results may not be generalised to all active ingredients with fungicide action. Mussen *et al*. (2004) revealed toxic effects of fungicides on larvae like captan, ziram or iprodione, while others active ingredients, i.e. cyprodinil, myclobutanil, trifloxystrobin, fenhexamid, and azoxystrobin did not show a larvicidal effect. Iprodione and captan were also found by Simon-Delso *et al*. (2014), iprodione being the second most-frequently observed fungicide (n = 13). Specifically, iprodione residues were detected in beekeeping matrices of morbid colonies (n = 9) and in healthy ones (n = 4) with levels between 0.017 and 1.5 ppm and 0.022-0.04 ppm, respectively. Zhu *et al*. (2014) showed larva toxicity of in hive levels of several pesticides including chlorothalonil. Furthermore, Chen *et al*. (2015) carried out a PER (proboscis extension reflex) test following the exposure during the larvae development with triadimefon and found that bees performed significantly worse at 0.4 ppm. The effects may be linked to the mode of action of the different active ingredients. Triadimefon is ergosterol biosynthesis inhibitor resulting in cell membrane disruption in fungi (PPDB, 2015), while boscalid affects the cell metabolism by inhibiting the enzyme succinate dehydrogenase (Stammler *et al*., 2008). Captan is a dicarboximide and disrupts the interactions with the sulfur moeity of glutathione, inhibiting the cell respiration (Roberts and Hutson, 1999). Iprodione is also a dicarboximide may additionally inhibit protein kinases affecting intracellular communication (Roberts and Hutson, 1999).

In field conditions, a co-exposure to different active ingredients or co-formulants exists. A number of studies showed the importance of this co-exposure in larval development (DeLorenzo and Serrano, 2003; Johnson and Percel, 2013; Zhu *et al.*, 2014). Specifically on fungicide formulations, Mullin (2015) describes an increase by up to 26.000-fold the toxicity for bees with regards to that of the active substance alone. A similar observation might be true for larval toxicity as well. In the present study we did not take this into account, but in the future or for risk assessment purposes, it may be interesting to study this hypothesis and test in parallel at least the active ingredient together with a number of formulated products or other active ingredients most frequently found in beekeeping matrices.

In conclusion, the active ingredient boscalid, at the level of exposure observed in field conditions, seems not to be lethal to worker honey bee larvae. Based on the study carried out, however, we cannot pronounce ourselves about potential effects on the adult bees emerged from these exposed larvae or about the potential impact on queen larvae. Furthermore, it would be necessary to evaluate the potential impact of boscalid alone or in combination with other products on adult bees (Sgolastra *et al*., 2016). Considering the persistency of boscalid in the field, an interesting approach would be to combine both methodologies: chronic exposure during larval and emerged-adult bees. However, the present results need to be considered in light of the latest findings of increased toxicity of pesticide formulations with regards to the active ingredients alone or pesticide mixtures.

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Received May 31, 2016. Accepted January 24, 2017.