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Report of the international Ring-Test for the Standardisation of an Acute Oral and Contact Test on Bumblebees in the Laboratory

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FOREWORD

This document contains the report of an international ring-test on the Bumblebee acute contact and oral toxicity studies (OECD Test Guidelines 246 and 247 respectively). The project was led by the Netherlands. The ring-test report was endorsed by the Working Group of the National Coordinators of the Test Guidelines Programme in April 2017 and the TG 246 and TG 247 were approved and are expected to be published in September 2017.

The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to the declassification of the report on 10th July, 2017. This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

REPORT OF THE INTERNATIONAL RING-TEST FOR THE STANDARDISATION OF AN ACUTE ORAL AND CONTACT TEST ON BUMBLEBEES IN THE LABORATORY

This report was endorsed by the 29th Meeting of the WNT in April 2017.

INTRODUCTION

1. A number of methods for acute toxicity testing of chemicals on bumblebees (*Bombus spp.*) have been published over the last years (e.g. van der Steen 1994 and 1996). The methodologies developed for bumblebee workers in the laboratory are based on the established methods for honeybee toxicity testing and allow for determining the acute oral and/ or contact LD_{50}. Since then, the test methods have been adopted and modified several times but no agreed testing protocol exists, as now of.

2. In February 2015, the international ICPPR Non-Apis working group held a workshop in Limburgerhof (Germany). During this workshop, two ring-test protocols for acute contact (single and group housing) and one for acute oral (single housing) laboratory testing were developed.

3. 17 laboratories from 10 countries representing academia, contract research institutes, chemical industry and governmental institutions contributed to the ring test in 2015. In this report, experimental results from 17 contact tests and 15 oral tests are presented.

PARTICIPANTS OF THE RING TEST GROUP

4. In total, 17 laboratories from 10 countries with different backgrounds, i.e. governmental institutions, universities, chemical industries and contract research institutes participated in the ring test. The regional background of these institutions was as follows: Belgium (1), Canada (1), France (2), Germany (6), Great Britain (2), Italy (1), the Netherlands (1), Spain (1), Switzerland (1), United States (1).

5. The names of the participating laboratories and contact details are given in the table below.

Contact person	**Laboratory**	**Email contact**
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Kristin Amsel	BioChem Agrar GmbH	Kristin.Amsel@biochemagrar.de
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6. In order to avoid any conflict of interest associated with disclosure of the names of laboratories that participated in this ring test, all names were anonymised using the following format: “LX", where “L" stands for “laboratory" and “X" indicates the number of that laboratory (out of 17 participants).

TIME SCHEDULE

Activity	Date
ICPPR NON-Apis group Workshop: for participating laboratories to organize and discuss details of the ring test.	February 2015 in Limburgerhof / Germany
Beginning of experimental phase End of experimental phase	May 2015 September 2015
Evaluation of results and presentation to the ring-test group	September 2015 – March 2016

MATERIAL AND METHODS

General

7. Details provided in the subsections of section 2.1 apply to both of the test methods, i.e. acute contact and acute oral.

Test item

8. Commercially available formulation of Dimethoate (EC 400).

Test species

9. The contact acute toxicity test for bumblebees (BBs) was conducted using workers of *Bombus* spp. as representative test species.

Colonies

10. Medium-sized colonies with brood in all stages of development and with a laying queen, each containing ~60-80 BB workers, were used in the test. Colonies should have been used in this ring-test within one week since the delivery date.

Acclimatisation

11. BBs were acclimatised to the test conditions overnight (12 – 24 h), with *ad libitum* access to an untreated aqueous 50% sucrose solution.

Climatic conditions

12. 25 ± 2°C, 60 ± 10% relative humidity, darkness.

Collection and randomization

13. Subject to the preferred approach typically used in a given laboratory, BB workers were collected from the colony either under red light (not anesthetised) or anaesthetised with CO_2 before they were transferred to group housing cages or to the pre-weighed Nicot cages. The participants agreed that very small and particularly very large BBs should have been excluded from the test by visual inspection. BBs entering the test were weighed individually. BB weight was used as gauge of an even distribution of BB sizes among the different treatment groups. Subsequently BBs were randomised onto the different treatments. Each BB was to be weighed before feeding.

Anaesthesia

14. In the acute contact test, BBs were sedated with CO_2 prior to application of the test and control solutions in order to prevent the droplets from being shed off due to movements of the workers.

15. Sedation of BBs was not mandatory in the acute oral test; however, some laboratories anaesthetised the workers with CO_2 to facilitate handling during the transfer from colonies into the individual cages.

Diet

16. The diet consisted of a 50% (w/v) aqueous sucrose solution. No pollen was administered to the BBs during the test.

Acute Contact Test

17. Details provided in the subsections of section 2.2 apply to the acute contact method only.

Test design

18. The test was conducted as a dose-response test, with five test item treatment groups and one water control treatment. Each treatment group consisted of 30 BBs.

19. Single housing: 30 BBs per treatment group and each BB placed into an individual cage for the duration of the test. Nicot queen breeding systems (see pictures attached) with 2mL syringes with cut-off tips to enlarge the feeding opening for the BBs were used.

Overview of the treatments:

Treatment	Description
1	untreated control
2	1.25 µg a.i. / bee Dimethoate
3	2.5 µg a.i. / bee Dimethoate
4	5 µg a.i. / bee Dimethoate
5	10 µg a.i. / bee Dimethoate
6	20 µg a.i. / bee Dimethoate

Application of the test item

20. The BBs were randomly assigned to each of the treatment groups. Anaesthetized BBs were weighed and then individually treated by topical application. 2 µl of solution containing the test item with a suitable dose were applied to the dorsal side of the thorax of each BB (between neck and wing base) using a micro-applicator. After the application, BBs were housed individually in Nicot cages and supplied with a 50 % aqueous sucrose solutions *ad libitum*.

Preparation of the test item doses and control

21. The test item was dissolved in water. 0.1% Triton X-100 was added to the solution serving as surfactant. Water control contained the same amount of surfactant (Triton X-100) as the test item treatments.

Assessments

22. Assessments of mortality were done either under the day or red light at 4-5h, 24, 48, 72 and 96h start after exposure.

23. Sublethal effects were recorded as follows:

- unaffected – BBs show inconspicuous behaviour;
- affected - BBs still upright and attempting to walk but showing signs of reduced coordination or hyperactivity;
- moribund (knock down) - BBs cannot walk and show only very feeble movements of legs and antennae, with only weak response to stimulation; e.g. light or blowing; such BBs may recover but usually die.

Acute Oral Test

24. Details provided in the subsection of section 2.3 apply to the acute oral method only.

Test design

25. The test was conducted as a dose-response test, with five test item treatment levels and a control treatment. Each treatment group consisted of 30 individually housed/caged BBs.

Overview of the treatments:

Treatment	Description
1	untreated control
2	0.25 µg a.i. / bee Dimethoate
3	0. 5 µg a.i. / bee Dimethoate
4	1 µg a.i. / bee Dimethoate
5	2 µg a.i. / bee Dimethoate
6	4 µg a.i. / bee Dimethoate

Feeding method

26. 40 µL diet was fed individually to each BB during the exposure phase of the test. During the observation period, diet was fed *ad libitum*. The BBs were fed via syringes plugged into the Nicot cages. The test system was kept horizontally, slightly inclined to the end of the Nicot cages (see pictures). Syringes were weighed before and after the exposure in order to determine the exact diet consumption.

Starvation

27. All BBs were starved for 2 to 4 hours prior to the exposure.

Exposure

28. The feeding period started when the syringes with the treatment-specific diet were plugged into the Nicot-cages and ended when these syringes were replaced with syringes containing untreated diet. The maximum duration of the exposure period was 4 hours.

Preparation of the test item doses and control

29. All feeding solutions were prepared using a 50 % aqueous sucrose solution. For the test item treatments, the sugar solution contained the respective amount of Dimethoate to meet the intended exposure level. The water control treatment consisted of an untreated 50 % aqueous sucrose solution with the appropriate amount of water.

Assessments

30. Assessments of mortality were done either under the day or red light at 4-5h, 24, 48, 72 and 96h start after exposure.

31. Sublethal effects were recorded as follows:

- unaffected – BBs show inconspicuous behaviour;
- affected - BBs still upright and attempting to walk but showing signs of reduced coordination or hyperactivity;
- moribund (knock down) - BBs cannot walk and show only very feeble movements of legs and antennae, with only weak response to stimulation; e.g. light or blowing; such BBs may recover but usually die.

32. The food consumption per bumblebee was calculated by weighing the syringes before and after the exposure.

Data analysis

33. During the analysis, data from each experiment were split into subsets corresponding to 24, 48, 72, and 96 h after exposure. For each of these subsets, a dose-response model was fitted, followed by the calculation of LD_{50} and the respective 95 % confidence limits.

Oral Test

34. When fitting a nonlinear regression model, the actual doses consumed where averaged for all bees per dose group, and mortality (treated as binary variable) was expressed as proportion of dead individuals from the total number of bees in that group. The property of the outcome was accounted for by incorporating the information on the actual sample size, i.e. the numbers of dead and alive bees in each group (Ritz and Streibig 2005, 2015).

35. Four dose-response functions as defined in Ritz (2010) were found to properly fit the analysed subsets per laboratory: 1) two-parameter log-logistic function, 2) three-parameter log-logistic function with the maximum response level fixed at 1, 3) two-parameter Weibull function of "type 1", and 4) three-parameter Weibull function of "type 2" with the maximum response level fixed at 1.

36. All four candidate models were fitted to each of the dose response relations generated by the different laboratories, and then the best single model was selected based on the Akaike Information Criterion, AIC (Akaike 1974; OECD 2006). The 95 % confidence intervals for the resultant estimates of LD_{50} were calculated according to the "delta method" (Casella and Berger 2002) as implemented in the drc software package (Ritz and Streibig 2005, 2015; see also subsection 0).

Contact Test

37. Dose-response models for the contact test data were fitted based on the nominal doses. Similar to the oral tests, different models were used to fit the data obtained in the contact tests, depending on the time after exposure and on the laboratory under consideration. In addition to the models listed in section 0, the following models were also used to fit the contact test data (Ritz 2010): 1) three-parameter Weibull function of "type 1" with the maximum response level fixed at 1, 2) four-parameter Weibull function of "type 2". Similar to the analysis of oral test data, selection of the optimal model for a given laboratory/time subset was based on the Akaike Information Criterion, AIC (Akaike 1974; OECD 2006). The 95 % confidence intervals for the resultant estimates of LD_{50} were calculated according to the "delta method" (Casella and Berger 2002) as implemented in the drc software package (Ritz and Streibig 2005, 2015; see also subsection 0).

Software Used

38. All calculations presented herein were conducted in the R v3.1.0 statistical computing environment (R Core Team 2014). Dose-response models were fitted using the add-on package *drc* v2.3-96 for R (Ritz and Streibig 2005, 2013). Illustrations were prepared either with the help of the standard R's graphical functions or using the functionality of the package *ggplot*2 v1.0.0 (Wickham 2009; Wickham and Chang 2014). All R scripts are available as part of this report and can be used to fully reproduce the presented results.

RESULTS AND DISCUSSION

Contact Test Results

39. Contact test data generated according to the agreed protocol were submitted by a total of 16 laboratories. All of these data are presented in the results described below.

Mortality in the control and test item treatments

40. 96 h after start of exposure phase control mortality of the bumblebees was on average 3,1 %, varying between 0 % (8 experiments) and 23.3 % (1 experiment). The mean LD_{50} for dimethoate was 4 µg a.i. / BB ranging between 1,05 and 10.07 µg a.i. / BB (Figure 1). Individual data from each laboratory are shown Table 1.

41. Mortality induced by the test item occurred predominately in the first 24 h. Consequently, the initial level of LD_{50} values at 24 h remained stable during the observation period until the end of the test, 96 h after exposure (Figure 2).

Table 1: Cumulative mortality in [%] in the control and different test item treatments and the LD_{50} values after 96 h

Treatment	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	L11	L12	L13	L14	L15	L16	Mean
Control treatment:																	
0	3.3	0	0	3.3	0	0	6.7	3.3	0	3.3	0	0	3.3	0	23.3	3.3	3.1
Test Item treatments: Dimethoate (EC 400)																	
1.25	3.3	3.3	0	3.3	23.3	0	10	26.7	3.3	60	0	16.7	6.7	0	76.7	13.3	15.4
2.5	6.7	20	6.7	13.3	86.7	33.3	30	60	20	93.3	3.3	20	16.7	3.3	100	13.3	32.9
5	60	53.3	53.3	56.7	96.7	56.7	93.3	100	50	100	70	46.7	76.7	40	100	23.3	67.3
10	86.7	96.7	96.7	100	100	86.7	100	100	83.3	100	96.7	96.7	96.7	80	100	50	91.9
20	100	100	100	100	100	93.3	100	100	100	100	100	100	100	96.7	96.7	93.3	98.8
LD_{50} values																	
LD_{50} [µg/BB]	4.7	4.6	4.8	4.7	1.6	3.9	3.3	2.1	4.8	1.1	4.1	4.4	3.9	5.6	1.1	10.1	4.0

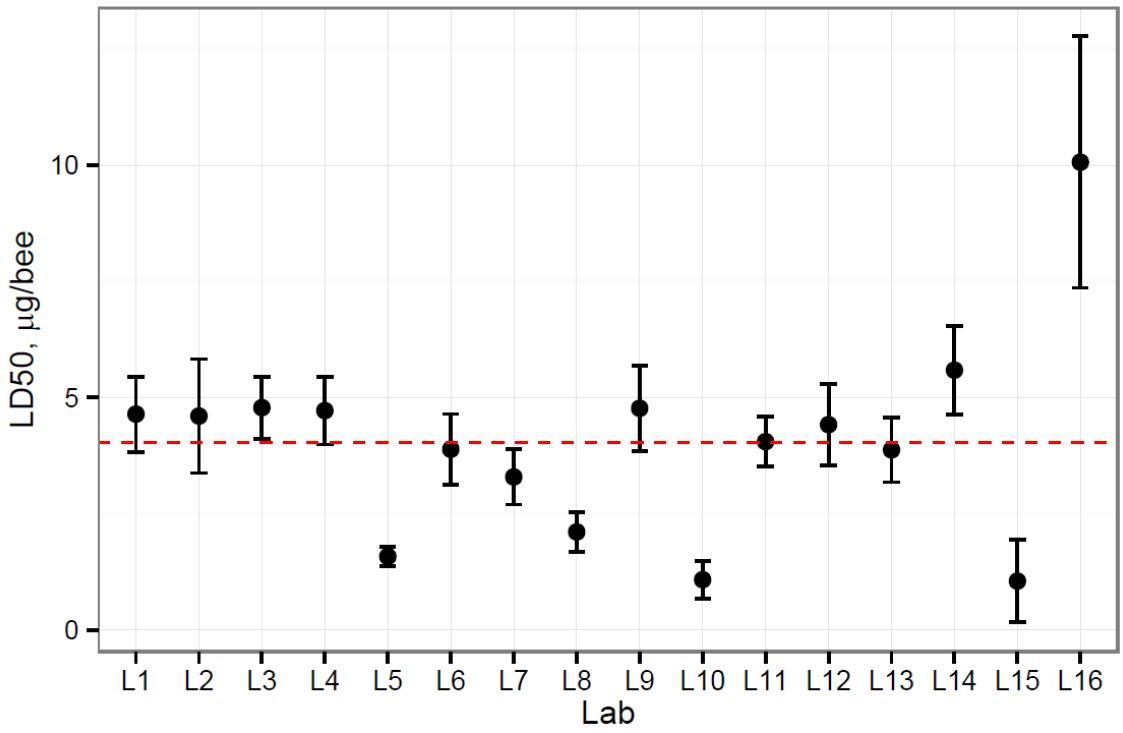

Figure 1: Inter-laboratory variation of the LD_{50} estimates obtained in 16 contact tests at 96 h after exposure. Vertical lines are the 95 % confidence intervals. The red horizontal line is the overall average LD_{50} [µg a.i. / BB].

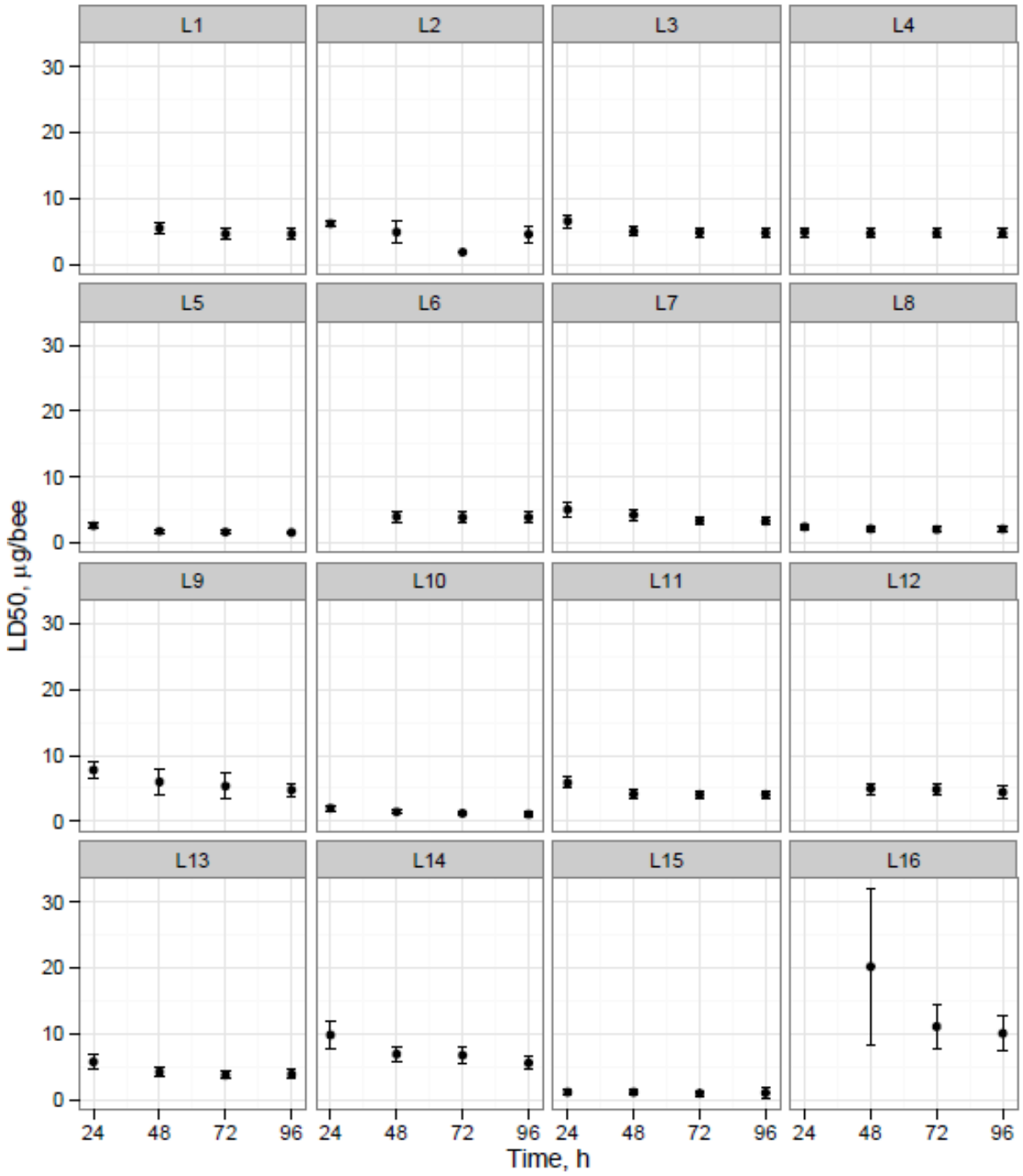

Figure 2: Point estimates of LD_{50} and their 95 % confidence intervals obtained in the contact tests at 24 (if possible), 48, 72 and 96 h after exposure. Data are shown for each laboratory in a separate subplot.

Effects of experimental parameters on the estimates of LD_{50} values

42. Although all contact tests were conducted by the participating laboratories according to a similar protocol, some experimental parameters varied among the laboratories (Table 4) and thus potentially could influence the resultant estimates of LD_{50}. Figure 3 displays the relationships between some of the recorded experimental parameters and 96 h LD_{50}. In most cases, these relationships seemed to be very week to moderate. It should be noted, however, that Figure 3 describes correlations with individual experimental parameters, while in reality the effects on LD_{50} might be determined by specific combinations of particular parameters. Therefore, the effects of experimental parameters on 96 h LD_{50} were tested simultaneously by fitting a linear regression model.

43. The analysis started from a model that included the average bee weight and a dummy variable for CO_2 application (i. e. a variable that takes a value of 1 for "yes" and 0 for "no"). These predictors were included into the initial model as they seemed to have the strongest individual relationships with LD_{50} Figure 3. The initial model was then reduced by removing predictors whose coefficients had the highest p-values. This stepwise analysis showed that the average bee weight was the only factor statistically significantly associated with 96 h LD_{50} (P = 0.029, t-test). The respective equation for this relationship was as follows (see also the blue regression line in Figure 3):

$$LD_{50} = -5.959 + 0.044 \text{ x Weight.}$$

44. It must be emphasized, however, that the revealed relationship between the bee body weight and LD_{50} has to be treated with great caution. As is seen from Figure 3, this relationship could be an artefact determined by the presence of one particularly high value of LD_{50} (10.068 µg a.i. / BB) recorded in L16 (see also Figure 1). A Dixon Q-test was performed on the set of LD_{50} values observed and the result from L16 (10.068 µg a.i. / BB) could be characterized as an outlier with 95 % confidence (N = 10, Q = 0.5). Indeed, when fitted without this observation, the relationship between the bee body weight and LD_{50} became insignificant (P = 0.198, t-test).

45. The available data show no strong evidence for relationships between the estimates of 96 h LD_{50} and any of the experimental parameters.

46. Overall mean body weights varied significantly among laboratories from 191.3 mg to 274.7 mg (Figure 4, P < 0:001, two-way ANOVA). However, the mean body weight in the different treatment groups was more or less consistent within most laboratories (Figure 5), with only few laboratories deviated from this general trend with significantly deviating bodyweights for at least one of the treatment groups (Figure 12, "dose group x laboratory" interaction (P < 0.001, two-way ANOVA)). The results of this analysis of variance suggest that some of the laboratories failed to correctly distribute the bumblebees in terms of their body weight across dose groups. Nevertheless, as Figure 5 shows there was a rise in mortality with increasing dose levels in all laboratories, usually with a distinct slope between treatment group 2.5 µg and 5 µg Dimethoate / BB.

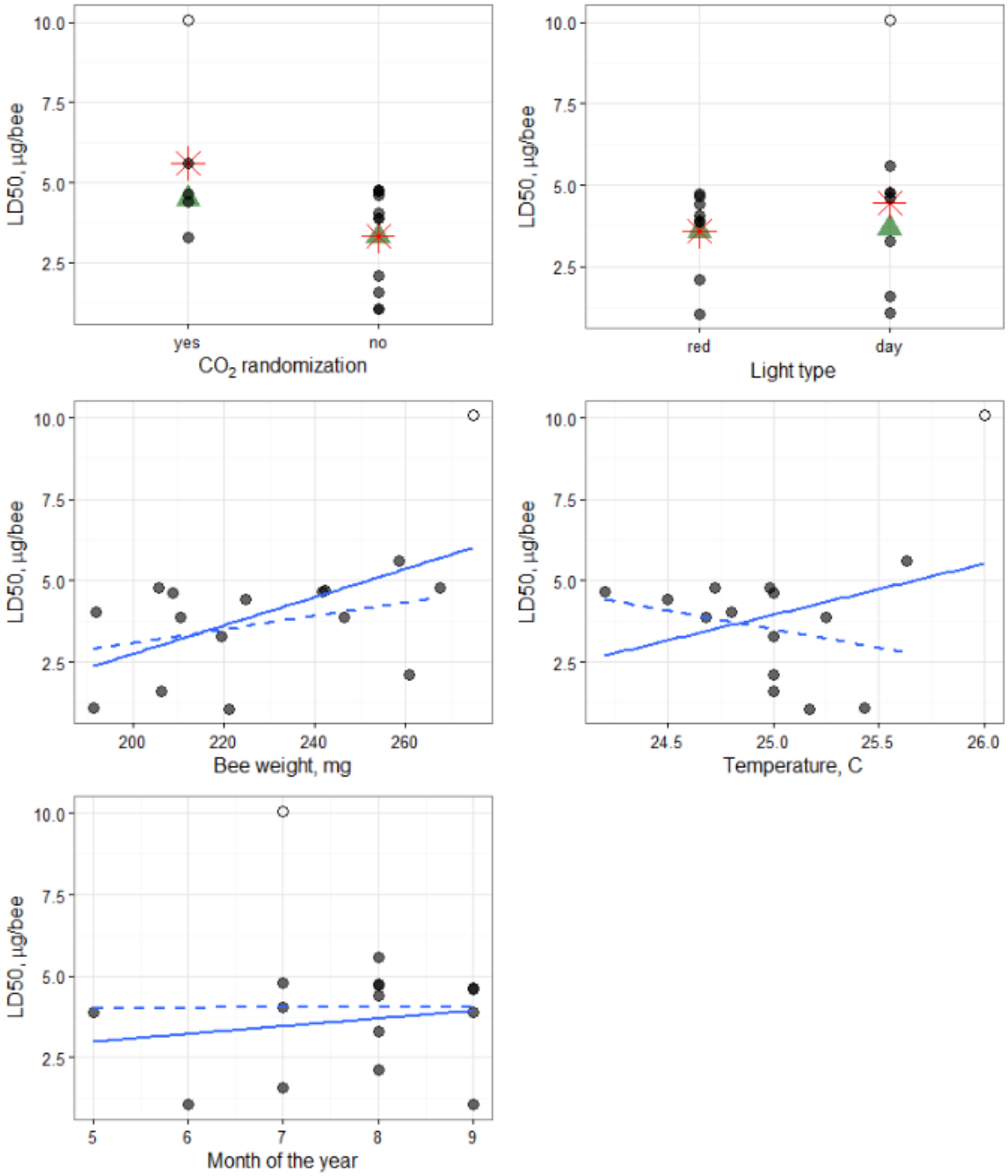

Figure 3: Relationships between the 96 h LD_{50} estimates obtained in 16 contact tests and some of the recorded experimental parameters. A blue regression line was added to the plot of average bee weight, temperature and month of the year to highlight the pattern. In the case of categorical variables, red asterisks were added to show the group means. The dashed blue regression line and the green triangle display pattern and group means when the outlier (transparent dot) was excluded.

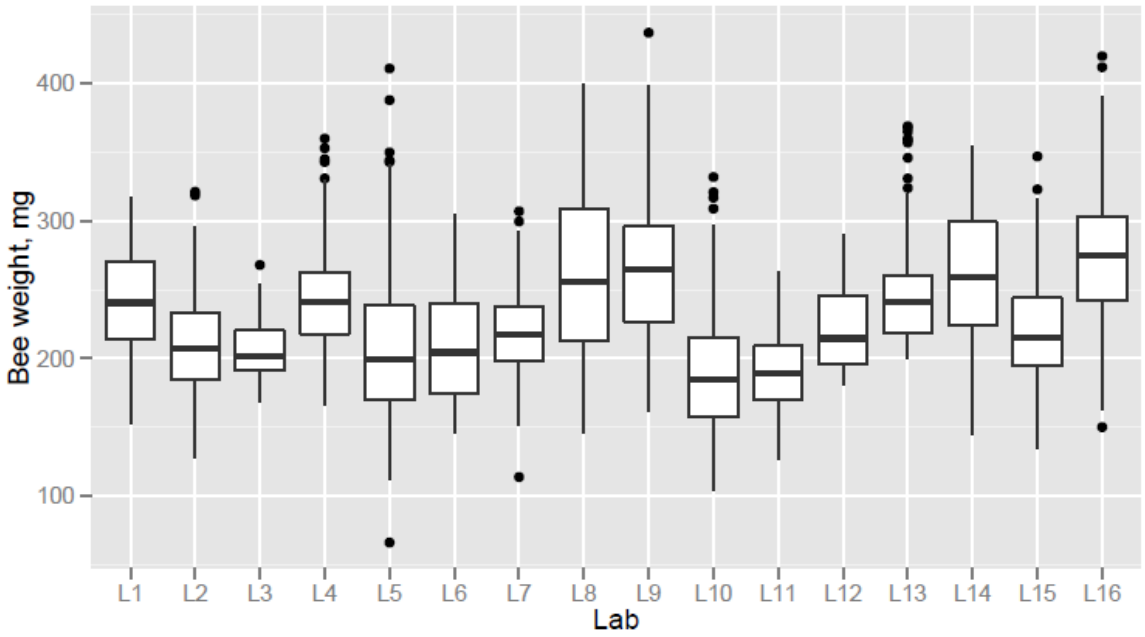

Figure 4: Distributions of the bee body weight in 16 contact tests conducted on bumblebees. The middle line in the box represents the median; box hinges (space from middle line to end of the box) represent the 1st and 3rd quartiles (box length covers 50 % of all observations, the interquartile range - IQR). The upper whisker extends from the hinge to the highest value that lies within the 1.53 IQR of the hinge. The lower whisker extends from the hinge to the lowest value that is within 1.53 IQR of the hinge. Dots are the data points that lie beyond within-the-whisker range of values.

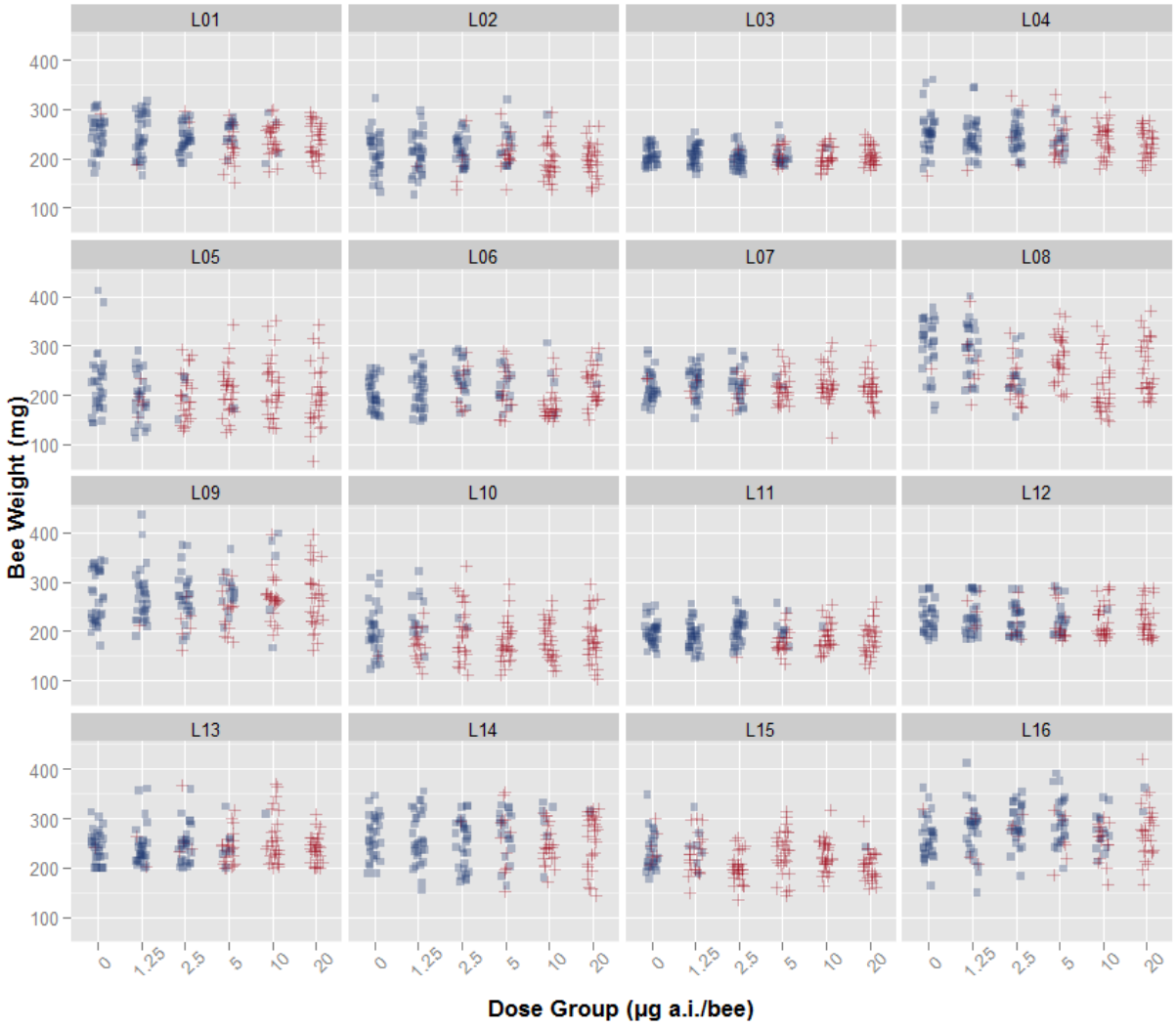

Figure 5: Distribution of body weight values of individual bumblebees allocated to the different dose groups in the contact trials. Blue squares represent bumblebees still alive at the end of the trial and red crosses represent dead individuals. Subplots correspond to each laboratory in which the trials were performed.

Oral Test

47. Oral test data were submitted by a total of 17 laboratories. Experiments from five laboratories were excluded from further analyses in this report: three laboratories did not record data on the consumption of the diet (i.e. did not weigh the feeders before and after the exposure phase) and thus no dose-response curve could be calculated.

48. Furthermore, no meaningful dose-response models could be fitted to the data from two other laboratories due to high mortalities across all dose levels of Dimethoate (0).

49. However, for completeness raw data from excluded experiments / laboratories are given in the Appendix (2.1.2)

Mortality in the control and the test item treatments

50. 96 h after start of exposure phase mean control mortality was 1.4 %, ranging from 0 % (9 laboratories) to 6.7 % (1 laboratory). The mean LD_{50} for dimethoate was 1 µg a.i./ BB, ranging between 0.5 and 1.8 µg a.i. / BB (Figure 6). Individual mortality data and LD_{50} values from each laboratory are shown in Table 2.

51. Mortality induced by the test item occurred predominately in the first 24 h. Consequently, the initial level of LD_{50} values at 24 h remained stable during the observation period until the end of the test, 96 h after exposure (Figure 7).

Table 2: Cumulative mortality in [%] in the control and different test item treatments and the LD_{50} values after 96 h

Treatment	L1	L2	L3	L4	L5	L6	L7	L8	L10	L11	L13	L14	Mean
Control treatment													
0	0	3.3	0	0	0	0	0	3.3	0	6.7	0	0	1.4
Test Item treatments: Dimethoate (EC 400)													
0,25	0	0	0	3.3	0	0	16.7	6.7	3.4	20	0	27.6	5.7
0,5	6.7	6.7	6.7	0	3.3	33.3	30	16.7	0	33.3	3.3	73.3	15.8
1	33.3	30	73.3	16.7	53.3	93.3	96.7	43.3	3.3	50	0	89.7	43.8
2	93.3	90	90	83.3	100	93.3	100	83.3	90	96.7	63.3	96.7	85.2
4	90	80	100	93.3	100	100	86.7	96.7	100	100	100	96.7	94.9
LD_{50} values													
LD_{50} [µg/BB]	1.2	1.0	0.8	1.2	0.9	0.5	0.5	1.1	1.6	0.9	1.8	0.4	1.0

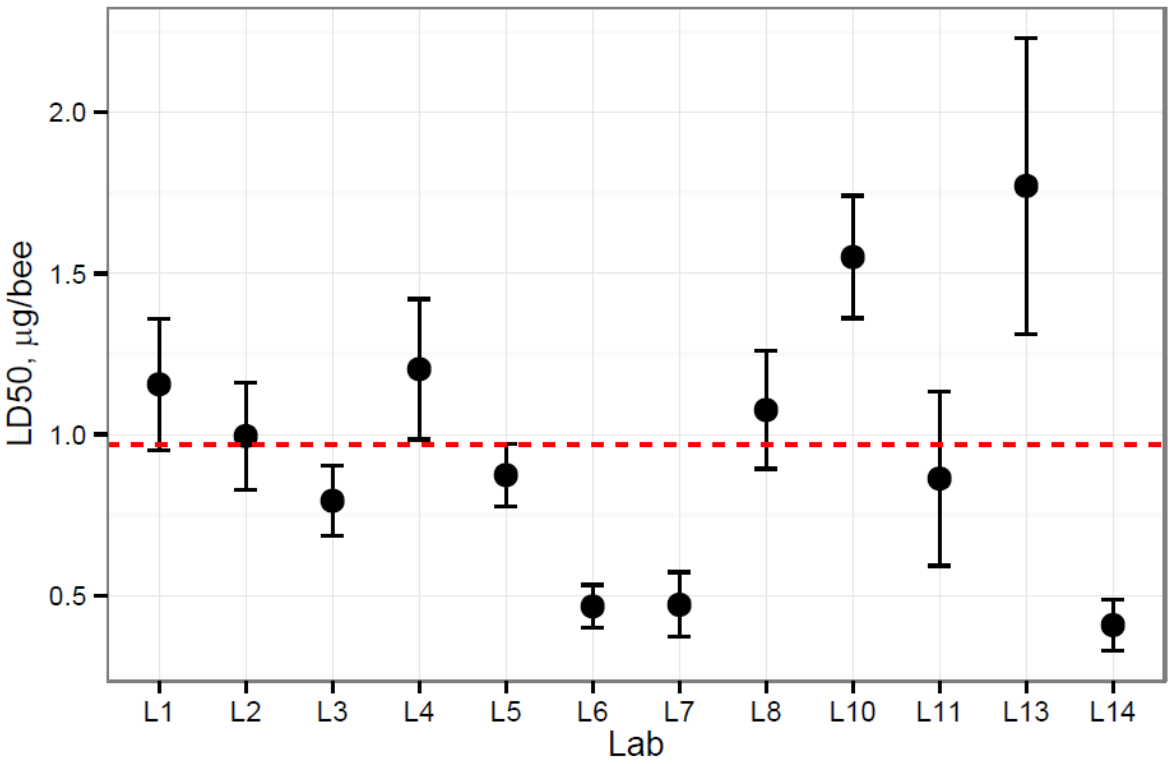

Figure 6: Inter-laboratory variation of the LD_{50} estimates obtained in the oral tests at 96 h after exposure (based on the average actual doses consumed by the bees). Vertical lines are the 95 % confidence intervals. The red horizontal line is the overall average LD_{50}.

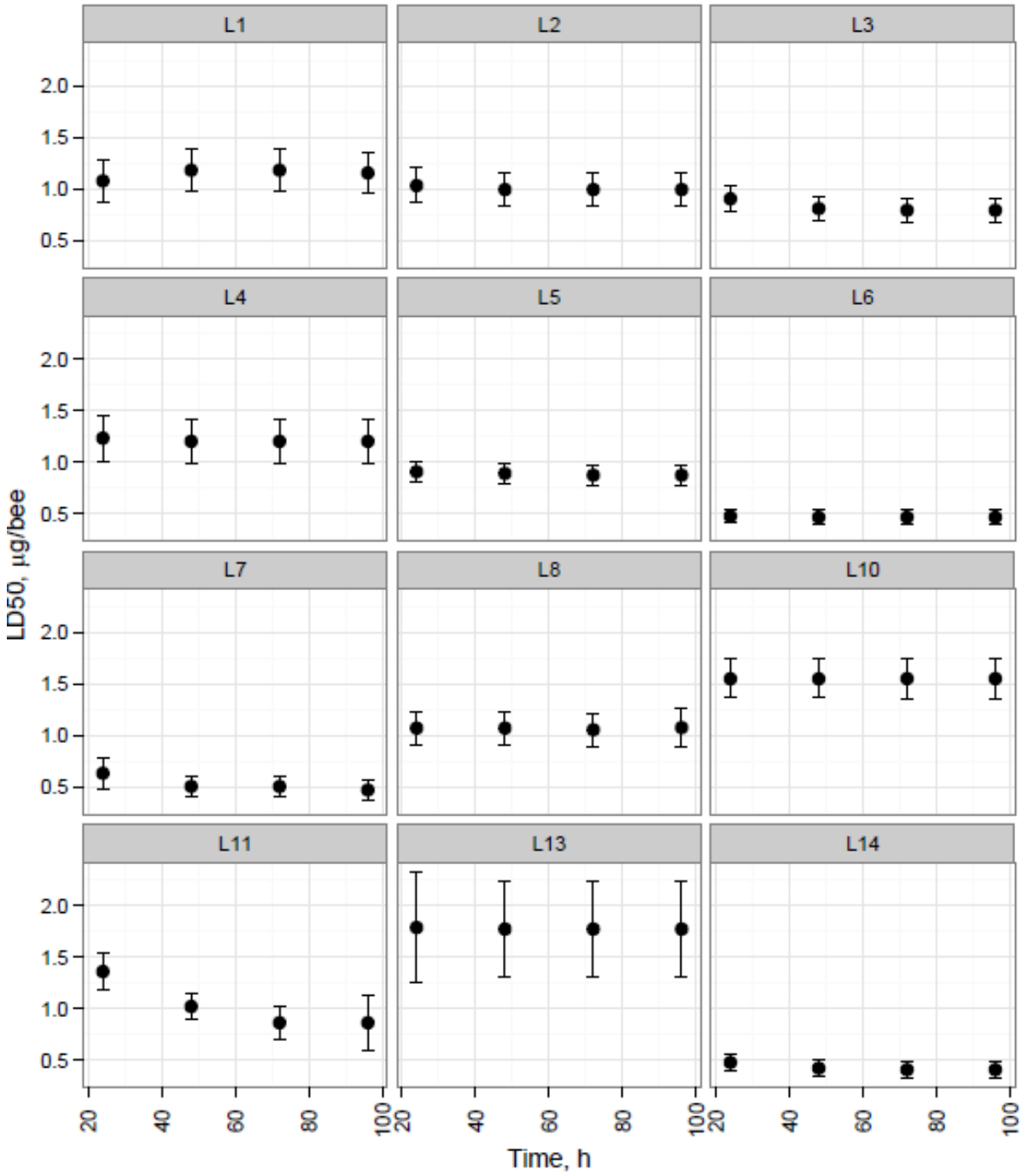

Figure 7: Point estimates of LD_{50} and their 95 % confidence intervals obtained in the oral tests at 24, 48, 72 and 96 h after exposure, based on the average actual doses consumed by the bees in each dose group. Data are shown for each laboratory in a separate subplot.

Effects of experimental parameters on the estimates of LD_{50} values

52. Although all oral tests were conducted by the participating laboratories according to a similar protocol, some experimental parameters varied among the laboratories and thus potentially could influence the resultant estimates LD_{50}. Figure 8 illustrates the relationships between the values of 96 h LD_{50} and some of the recorded experimental parameters. In most cases, these relationships seemed to be very week to moderate. It should be noted, however, that Figure 8 describes correlations with individual experimental parameters, while in reality the effects on LD_{50} might be determined by specific combinations of certain parameters. For example, there were laboratories randomizing the bees under the red light with CO_2 anaesthesia, and there were laboratories performing this under the red light without such anaesthesia (see Figure 8). These laboratories differed also with regard to other factors. Therefore, the effects of experimental parameters on the estimates of 96 h LD_{50} were tested simultaneously by fitting a linear regression model.

53. The analysis started from a model that included air temperature, bee weight, test date (i.e. number of the month) and CO_2 application (1 for “yes” and 0 for “no”). These predictors were included into the initial model as they seemed to have the strongest individual relationships with LD_{50} (Figure 8). Adding other predictors was not meaningful because of the low degrees of freedom available (i. e. for the 9 potentially interesting predictors only 12 data points were available for analysis). The initial model was then reduced by progressively removing predictors whose coefficients had the highest p-values.

54. This stepwise analysis showed that none of the analysed experimental factors were statistically significantly associated with the 96 h LD_{50}.

55. Overall mean body weights varied significantly among laboratories from 156.7 mg to 270.5 mg (Figure 9, $P < 0:001$, two-way ANOVA). However, the mean body weight in the different treatment groups was consistent within laboratories (Figure 10 & Figure 13, “dose group x laboratory" interaction ($P < 0.859$, two-way ANOVA)). The results of this analysis of variance suggests that all laboratories correctly distributed the bumblebees in terms of their body weight across dose groups. Additionally Figure 10 shows there was a rise in mortality with increasing dose levels in all laboratories, usually with a distinct slope between treatment group 1 µg and 2 µg Dimethoate / BB.

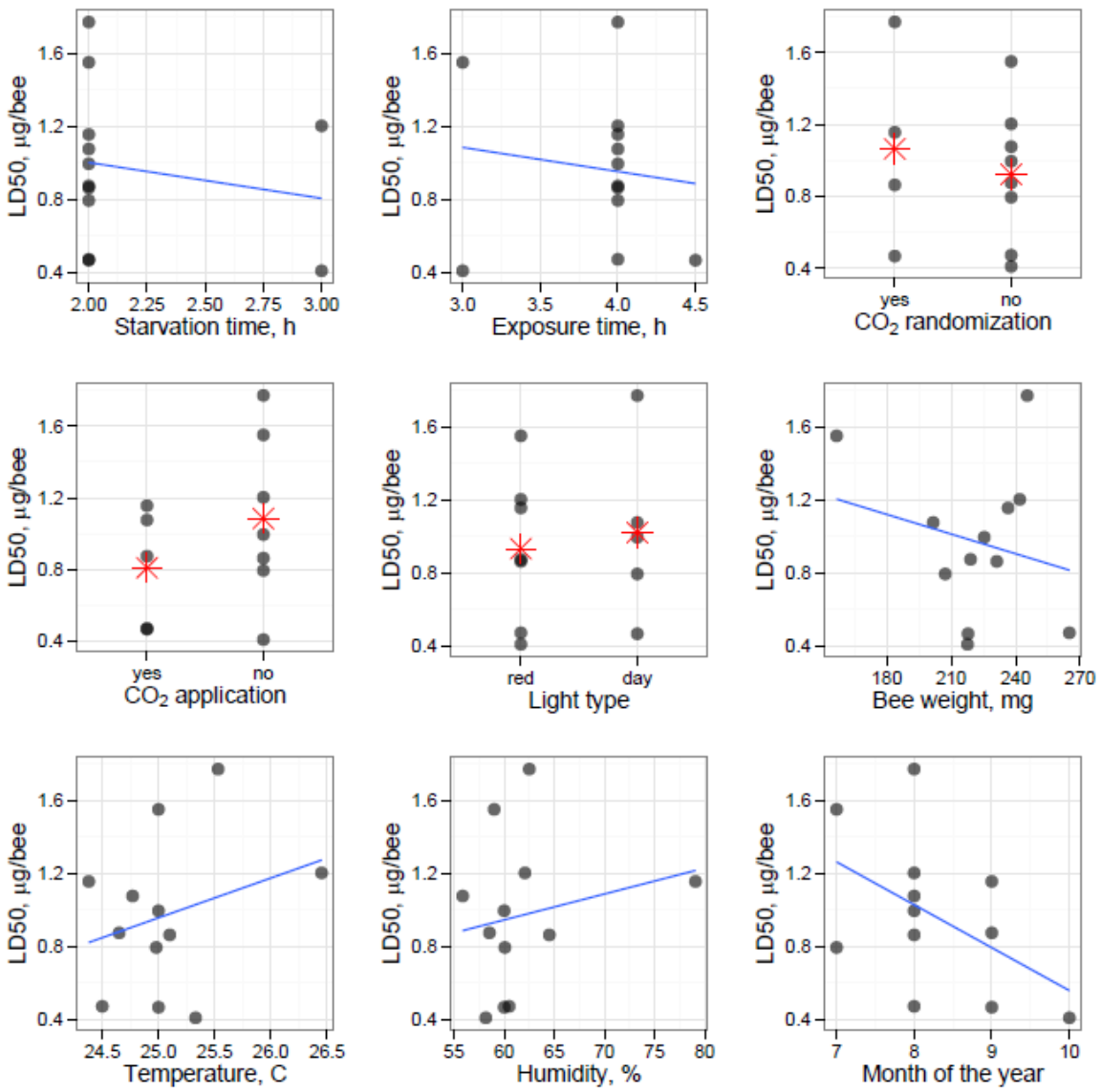

Figure 8: Relationships between the 96-h LD_{50} estimates obtained in 12 oral tests and some of the recorded experimental parameters. Regression lines (shown in blue) were added to highlight the patterns for numeric variables. In the case of categorical variables, red asterisks were added to show the group means.

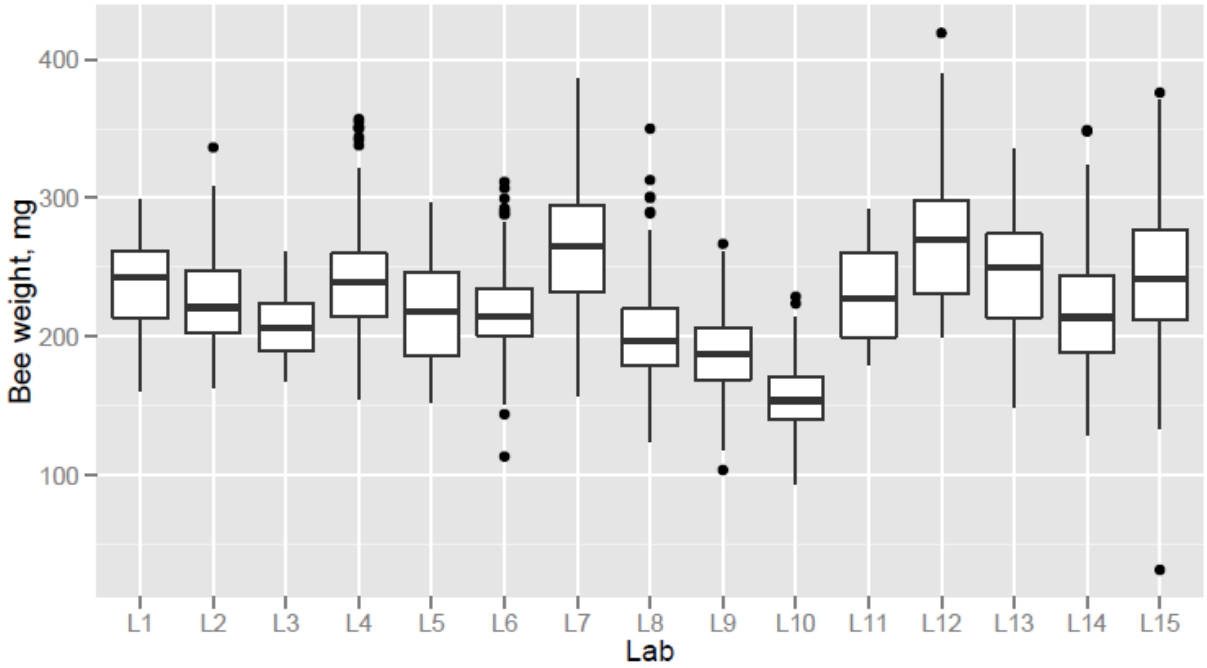

Figure 9: Distributions of the body weight in oral tests conducted in L1 to L15 (in this figure also L9, L12 and L15 are presented even if they did not record data on the consumption of the diet and were excluded from further analyses in this report). See Figure 4 for interpretation of this plot.

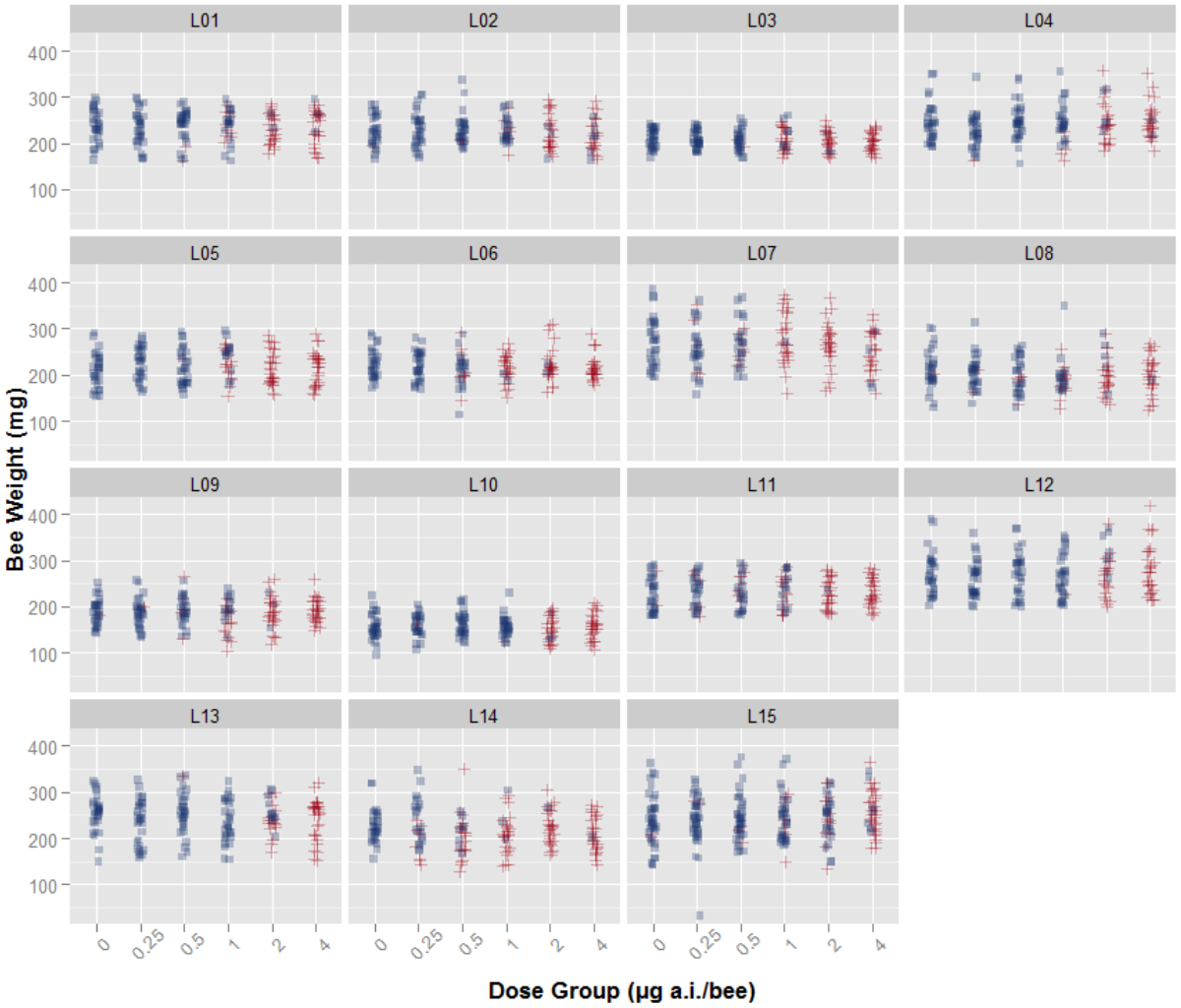

Figure 10: Distribution of body weight values of individual bumblebees allocated to the different dose groups in the oral trials. Blue squares represent bumblebees still alive at the end of the trial and red crosses represent dead individuals. Subplots correspond to each laboratory in which the trials were performed.

Actual food uptake and resulting exposure

56. All laboratories have individual bumblebees consuming less than 80 % of the average food uptake of the treatment group. Numbers are ranging from 1 bumblebee up to 30 bumblebees (Figure 11). It is considered that these individuals have not sufficiently fed and therefore not adequately been exposed to the test chemical for oral toxicity assessment purposes. In higher dose groups these so called "non-feeders" often survive in contrast to the individuals consuming more than 80 % of the average food uptake.

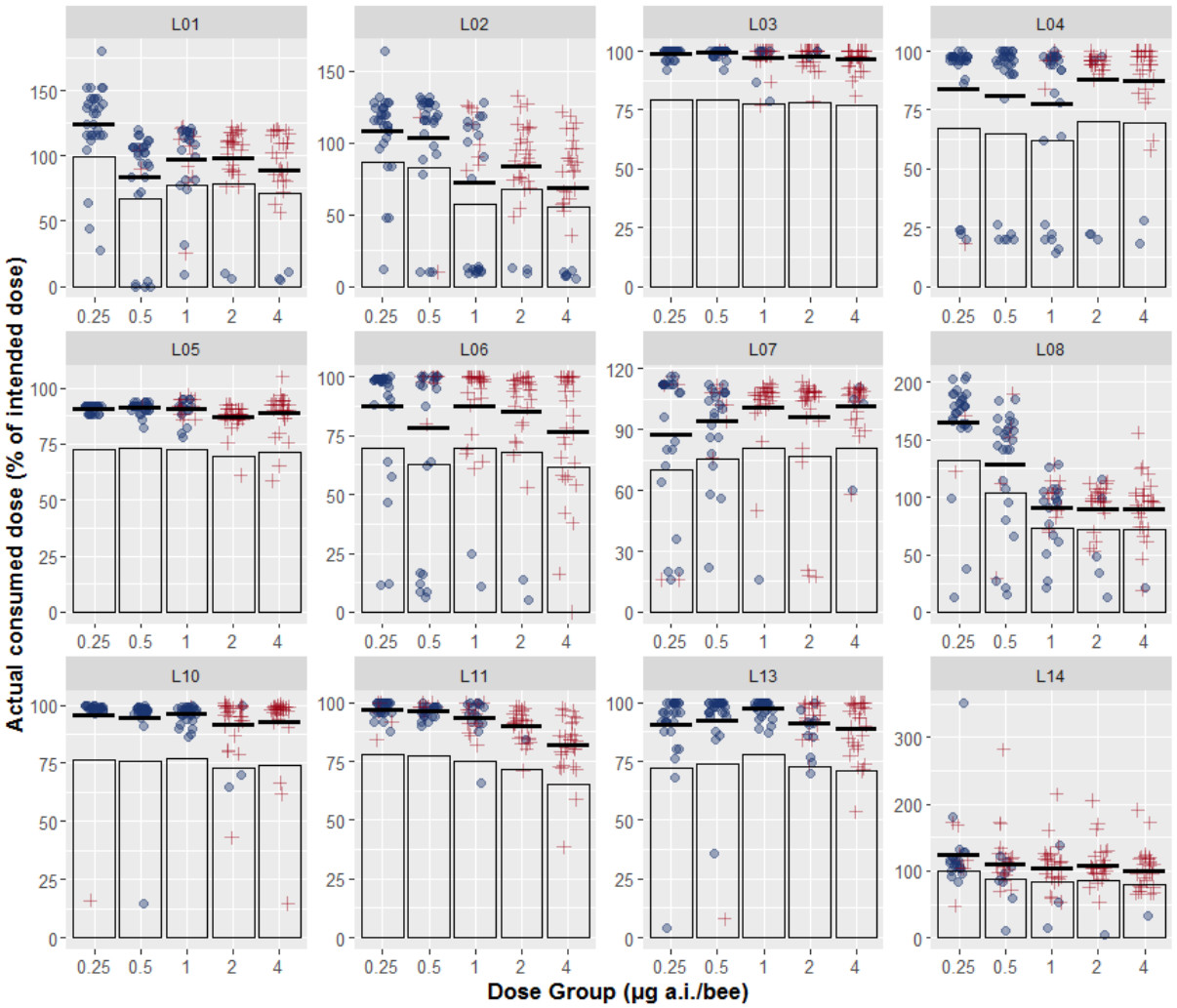

Figure 11: Relative dose consumption (% of intended dose) in the oral toxicity trials. Subplots correspond to each laboratory in which the trials were performed. Round points correspond to bumblebees still alive at the end of the trials and crosses represent dead individuals. Black horizontal lines represent the average oral uptake (%) for each dose group and the rectangles below them correspond to the range between 0 % and 80 % of these average values.

Additional Observations

57. Some laboratories set up additional treatment groups to identify auxiliary solvent concentrations and a dispersant especially needed for active ingredient testing due to solubility issues. Therefore, one treatment consisted of water with 10 % Acetone and one treatment of water with 0.1 % Rhodopol 23 (Xanthan Gum). Each treatment group consisted of 30 bumblebees, too.

58. 96h after start of exposure phase, mortality in the water control was 0 % in 5 experiments and 3.3 % in 1 experiment. The use of 10 % (v/v) acetone caused 0 % mortality in 5 experiments and 3.3 % in 1 experiment. 3 experiments with 0.1 % Rhodopol 23 were conducted, one producing 0 % mortality, one 3.3 % and one 13.3 %.

59. Individual data for each laboratory are shown in Table 3.

Table 3: Mortality of bumblebees in additional treatment groups in the oral test conducted by 6 laboratories (labelled with letters A to F for convenience) after 96 h

Laboratory	Test treatment	Mortality
A	Water 10 % Acetone 0.1 Rhodopol 23	0 % 0 % 3.3 %
B	Water 10 % Acetone 0.1 Rhodopol 23	0 % 0 % 0 %
C	Water 10 % Acetone	0 % 0 %
D	Water 10 % Acetone 0.1 Rhodopol 23	0 % 3.3 % 13.3 %
E	Water 10 % Acetone	3.3 % 0 %
F	Water 10 % Acetone	0 % 0 %

CONCLUSIONS

60. Overall, mortality was low in control groups in both contact and oral exposure, typically not exceeding 7 %. Only in one out of 16 conducted experiments, control mortality exceeded 10 % in the acute contact test. In the acute oral experiments, in all 17 tests (including the five experiments excluded due to reasons detailed in subchapter 3.2) control mortalities were below 10 %. Thus, both ring-tested methods showed to be feasible and reproducible. Based on the ring-test results a validity criterion of ≤ 10 % mortality for the untreated control treatment can be justified.

61. The results of this ring test show the described test methods are suitable to assess the acute effects of test chemicals on bumblebees in the laboratory.

62. The level of LD_{50} values at 24 h after exposure (contact & oral) remained stable during the observation period until the end of the test, suggesting that experiments can be stopped before 96 h, while still providing reliable information on the dose response for subsequent endpoint calculation.

63. Therefore, the general test duration will be 48 h. Only when corrected mortality increases by ≥ 10 % between 24 h and 48 h in at least one treatment group whilst control mortality remains at an accepted level, i.e. $\leq$10%, the duration of the test has to be extended up to 96 h.

64. None of the experimental parameters were conclusively found to be associated with the 96 h point estimates of LD_{50}. Although, LD_{50} is not influenced by body weight, it is recommended to exclude very small and particularly very large bumblebees from the test by visual inspection. Additionally, bumblebees of all sizes should be equally distributed among all treatment groups.

65. There was a considerable inter-laboratory variation in survival of the bumblebees, especially in the intermediate dose groups. At least partially, this variation could be explained by the degree of deviation of the actual doses consumed by individual bees from the respective nominal values. As one might expect, actual consumed doses that were considerably lower than the respective nominal values were often associated with lower mortality. Therefore, the acute oral bumblebee testing needs to account for so called "non-feeders". A "non-feeder" is an individual bumblebee that consumes < 80 % of the mean consumption of the respective treatment group. Due to the limited food uptake, "non-feeders" have not been sufficiently exposed to the test chemical and should therefore not be considered in the derivation of endpoints. Otherwise, the limited exposure of "non-feeders" could possibly lead to an overestimation of LD_{50} values.

66. To address low solubility of potential test items, additional treatment groups in the acute oral test identified feasible concentrations of the solvent acetone as well as the polysaccharide Rhodopol 23 (Xanthan Gum). Results showed that with respect to diet uptake and mortality 10 % acetone and 0.1 % Rhodopol 23 can be added to the diet. However, it depends on the test item whether these concentrations contribute to a homogeneous distribution in the diet.

APPENDIX

Detailed information on experimental parameters on the experiments conducted in the different laboratories

67. Table 4 provides a summary of the experimental parameters of the 16 acute contact tests conducted in this ring test. As is seen in this table, the contact tests were conducted between May and September 2015. The average temperature and humidity recorded in these experiments varied from 24.2 °C to 26.0 °C and from 55.7 % to 76.0 %, respectively. Five of the 16 laboratories used CO_2 randomization when collecting the bees from the colonies, and 15 of the 16 laboratories used CO_2 for anaesthesia. Eight of the 16 laboratories sorted the bumblebees under red light. Most of the laboratories used a sucrose solution as the food for bees (n = 13), two laboratories used apiinvert, and one laboratory used a sugar solution. Most laboratories used Triton X as the solvent for Dimethoate (n = 15), and only one laboratory used Sticman.

Table 4: Experimental parameters recorded in the 16 contact tests

Lab	Month	CO_2 randomization	CO_2 application	Light	Solvent	Food type	Average temperature (°C)	Average humidity (%)
L1	Sep	yes	yes	red	triton	apiinvert	24.2	76.0
L2	Sep	no	yes	day	triton	sucrose	25.0	60.0
L3	Jul	no	yes	day	triton	sucrose	25.0	60.1
L4	Aug	no	yes	red	triton	sucrose	not recorded	not recorded
L5	Jul	no	yes	day	triton	sucrose	25.0	60.0
L6	Sep	no	yes	red	triton	sucrose	24.7	59.3
L7	Aug	yes	yes	day	triton	sucrose	25.0	60.0
L8	Aug	no	yes	red	triton	sucrose	25.0	60.0
L9	Aug	no	yes	day	triton	sugar	24.72	55.9
L10	Jun	no	yes	day	triton	apiinvert	25.4	61.22
L11	Jul	no	yes	red	triton	sucrose	24.8	60.0
L12	Aug	yes	yes	red	triton	sucrose	24.5	60.5
L13	May	no	yes	red	sticman	sucrose	25.3	65.0
L14	Aug	yes	yes	day	triton	sucrose	25.6	55.7
L15	Sep	no	no	red	triton	sucrose	25.2	57.2
L16	Jul	yes	yes	day	triton	sucrose	26.0	not recorded

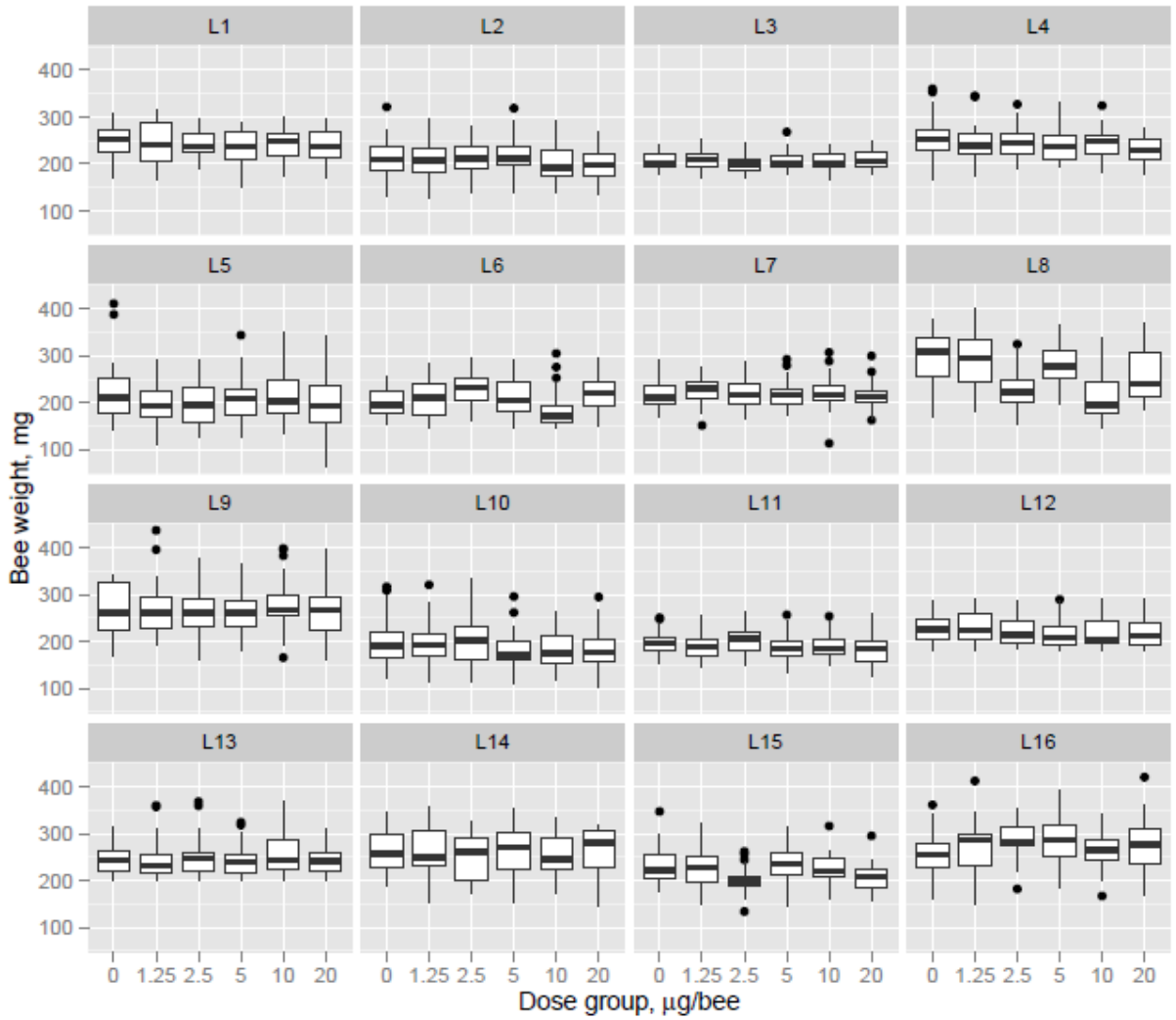

Figure 12: Distributions of the bees' body weight among different dose groups in 16 contact tests. See Figure 4 for interpretation of this plot.

68. As is seen in Table 5, oral tests were conducted during the period from June to October 2015. The starvation and exposure times made up on average 2.4 and 3.9 h, respectively. The average temperature and humidity recorded in these experiments varied from 24.4 to 26.0 °C and from 55.9 to 79.1 %, respectively. Seven of the 15 laboratories used CO_2 randomization, and 6 of the 15 laboratories used CO_2 application. Eight of the 15 laboratories sorted the bumblebees under red light.

69. According to the agreed test protocol, each of the six dose groups were supposed to include 30 bees. However, one laboratory (L15) used 40 bees per group. In addition, due to various reasons, some observations in some of the dose groups were missing from the submitted datasets. Nevertheless, such missing values were rare, and did not exceed one observation per dose group, suggesting a negligible impact on the results of analysis.

Table 5: Experimental parameters recorded in the 15* oral tests

Lab	Month	Starvation time (h)	Exposure time (h)	CO_2 randomization	CO_2 application	Light	Food type	Average temperature (°C)	Average humidity (%)
L1	Sep	2	4	yes	yes	red	apiinvert	24.4	79.1
L2	Aug	2	4	no	no	day	sucrose	25.0	60.0
L3	Jul	2	4	no	no	day	sucrose	25.0	60.1
L4	Aug	3	4	no	no	red	sucrose	26.4	62.1
L5	Sep	2	4	no	yes	red	sucrose	24.6	58.5
L6	Sep	2	4.5	yes	yes	day	sucrose	25.0	60.0
L7	Aug	2	4	no	yes	red	sucrose	24.5	60.5
L8	Aug	2	4	no	yes	day	sugar	24.8	55.9
L9	Jun	4	4	yes	no	day	sucrose	25.1	59.8
L10	Jul	2	3	no	no	red	sucrose	25.0	59.0
L11	Aug	2	4	yes	no	red	sucrose	25.1	64.5
L12	Jun	4	4	yes	no	red	sucrose	26.0	68.3
L13	Aug	2	4	yes	no	day	sucrose	25.5	62.5
L14	Oct	3	3	no	no	red	sucrose	25.3	58.2
L15	Aug	2	4	yes	yes	day	sucrose	26.0	not recorded

* Please note that L9, L12 and L15 were excluded from evaluation due to not measuring the actual food uptake.

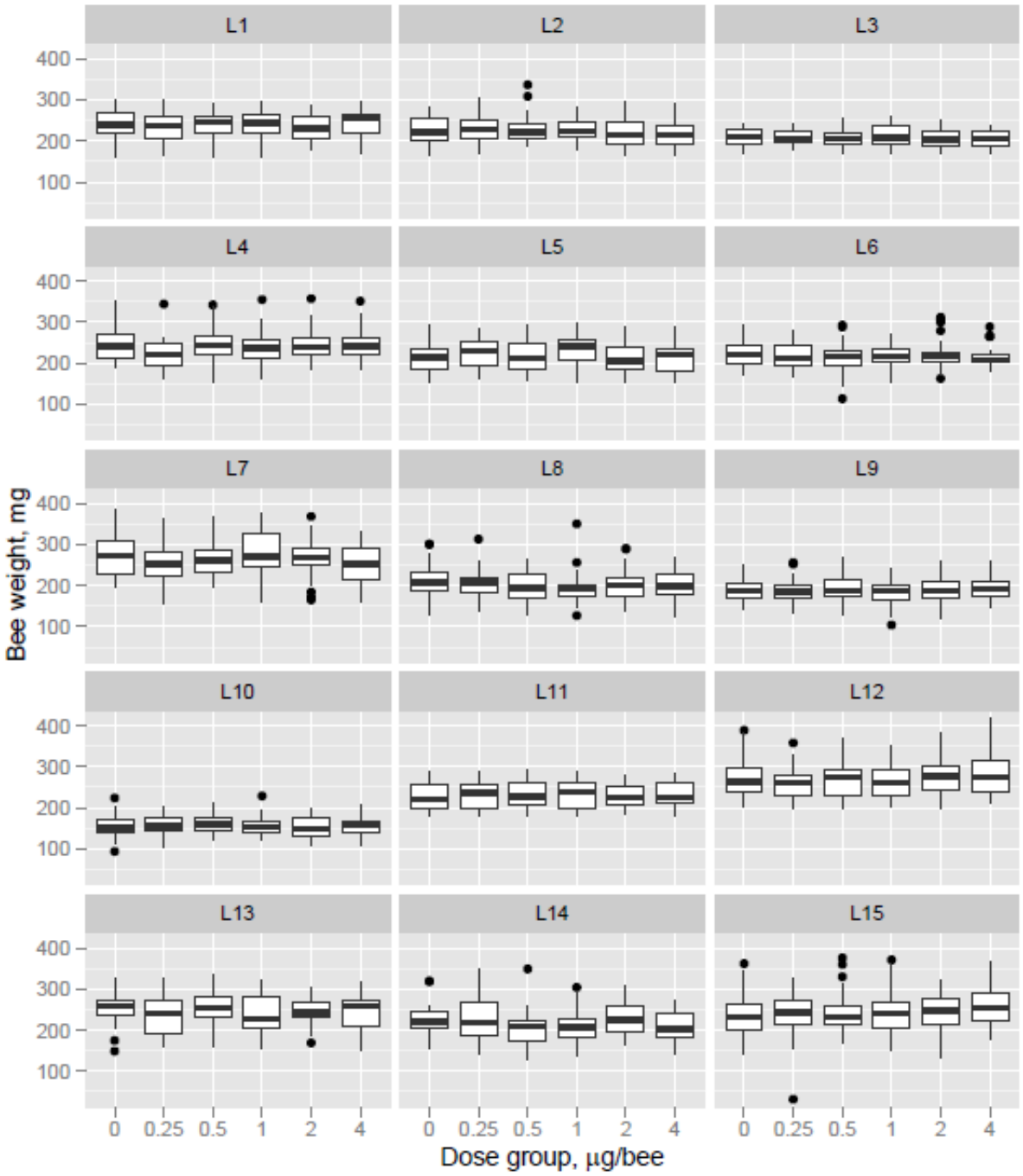

Figure 13: Distributions of the bees' body weight among different dose groups in 15* contact tests. See Figure 4 for interpretation of this plot.

* Please note that L9, L12 and L15 were excluded from evaluation due to not measuring the actual food uptake.

Summary of data from each laboratory

70. In the following data from the participating laboratories are given in more detail for the contact and oral test designs.

Summary of data in the acute contact tests

71. Summary of the data from 16 laboratories / experiments conducted according to the acute contact test design given in 0.

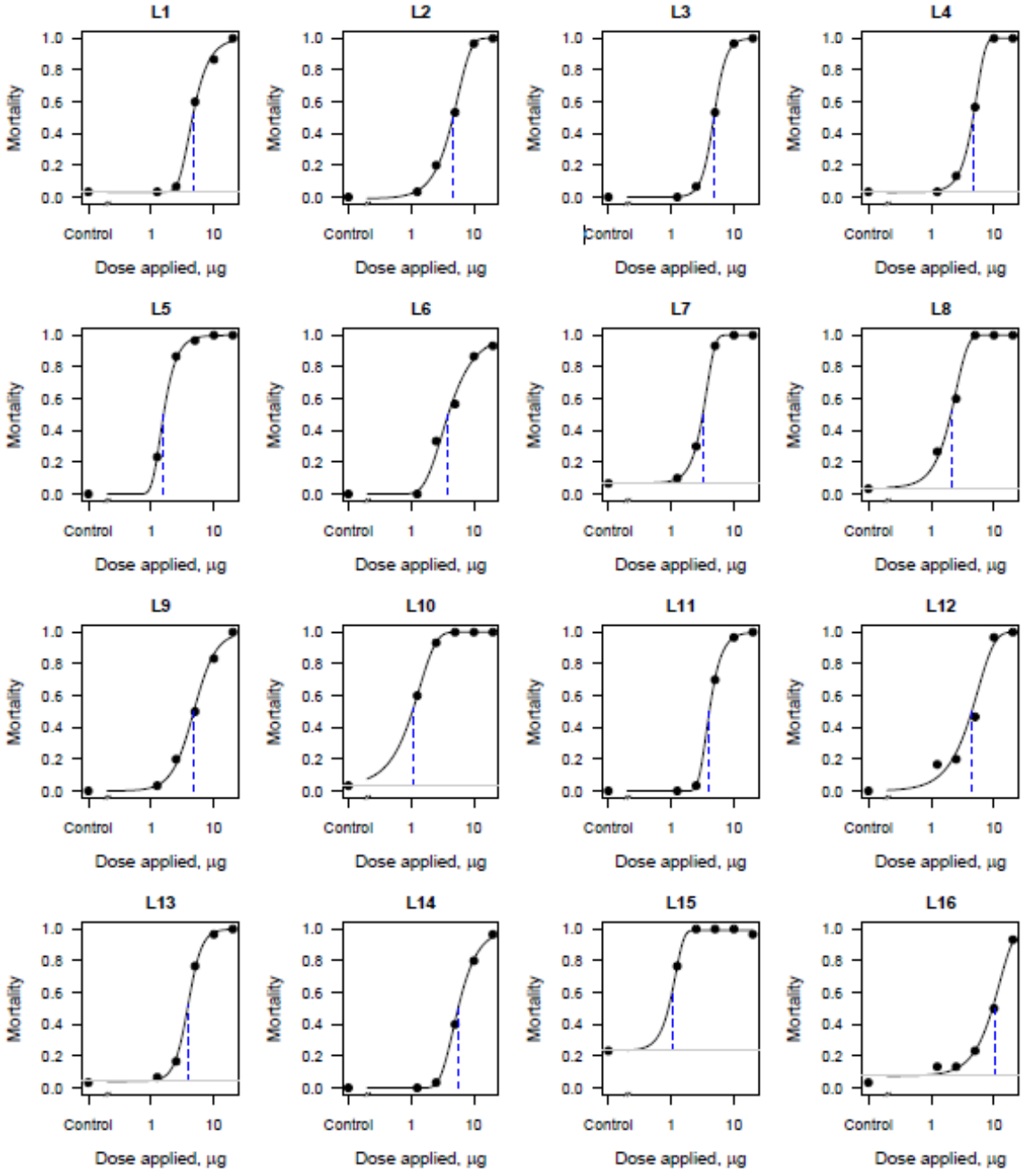

Figure 14: Dose-response models fitted to the contact test data obtained at 96 h after exposure (based on the nominal doses). The x-axis is on the log scale. The blue dashed lines denote LD_{50}. Note that in cases when mortality in control bees was estimated to be non-zero, these blue lines start from a non-zero level shown as a light grey horizontal line.

Table 6: Summary of mean mortality generated in the contact test by laboratory 1 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	1	1	1	1
1.25	0	1	1	1
2.5	2	2	2	2
5	3	14	18	18
10	22	26	26	26
20	30	30	30	30

Table 7: Summary of mean mortality generated in the contact test by laboratory 2 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
1.25	0	0	0	1
2.5	4	6	6	6
5	8	14	14	16
10	26	28	29	29
20	30	30	30	30

Table 8: Summary of mean mortality generated in the contact test by laboratory 3 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
1.25	0	0	0	0
2.5	1	2	2	2
5	6	14	15	16
10	26	29	29	29
20	30	30	30	30

Table 9: Summary of mean mortality generated in the contact test by laboratory 4 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	1	1	1	1
1.25	1	1	1	1
2.5	3	4	4	4
5	16	17	17	17
10	30	30	30	30
20	30	30	30	30

Table 10: Summary of mean mortality generated in the contact test by laboratory 5 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
1.25	1	3	5	7
2.5	15	25	26	26
5	27	29	29	29
10	30	30	30	30
20	30	30	30	30

Table 11: Summary of mean mortality generated in the contact test by laboratory 6 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
1.25	0	0	0	0
2.5	5	10	10	10
5	16	17	17	17
10	23	25	26	26
20	27	28	28	28

Table 12: Summary of mean mortality generated in the contact test by laboratory 7 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	1	2	2	2
1.25	2	3	3	3
2.5	6	7	9	9
5	16	20	27	28
10	27	30	30	30
20	30	30	30	30

Table 13: Summary of mean mortality generated in the contact test by laboratory 8 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	1
1.25	3	7	8	8
2.5	15	17	18	18
5	28	30	30	30
10	30	30	30	30
20	30	30	30	30

Table 14: Summary of mean mortality generated in the contact test by laboratory 9 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
1.25	0	1	1	1
2.5	2	5	6	6
5	5	13	14	15
10	20	23	24	25
20	29	30	30	30

Table 15: Summary of mean mortality generated in the contact test by laboratory 10 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	1	1
1.25	7	12	16	18
2.5	19	25	27	28
5	27	30	30	30
10	30	30	30	30
20	30	30	30	30

Table 16: Summary of mean mortality generated in the contact test by laboratory 11 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
1.25	0	0	0	0
2.5	1	1	1	1
5	10	21	21	21
10	27	28	29	29
20	30	30	30	30

Table 17: Summary of mean mortality generated in the contact test by laboratory 12 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
1.25	2	2	3	5
2.5	5	5	5	6
5	9	13	13	14
10	26	29	29	29
20	30	30	30	30

Table 18: Summary of mean mortality generated in the contact test by laboratory 13 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	1	1	1
1.25	1	2	2	2
2.5	2	3	4	5
5	12	20	23	23
10	24	28	29	29
20	30	30	30	30

Table 19: Summary of mean mortality generated in the contact test by laboratory 14 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
1.25	0	0	0	0
2.5	1	1	1	1
5	6	9	10	12
10	15	22	22	24
20	25	29	29	29

Table 20: Summary of mean mortality generated in the contact test by laboratory 15 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	3	5	6	7
1.25	18	20	23	23
2.5	29	30	30	30
5	30	30	30	30
10	30	30	30	30
20	29	29	29	29

Table 21: Summary of mean mortality generated in the contact test by laboratory 16 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	1	1	1	1
1.25	2	3	4	4
2.5	1	4	4	4
5	5	7	7	7
10	6	11	14	15
20	7	15	24	28

2.1.1 Summary of data in the acute oral test

Summary of the data from 12 laboratories / experiments conducted according to the acute oral test design given in 0.

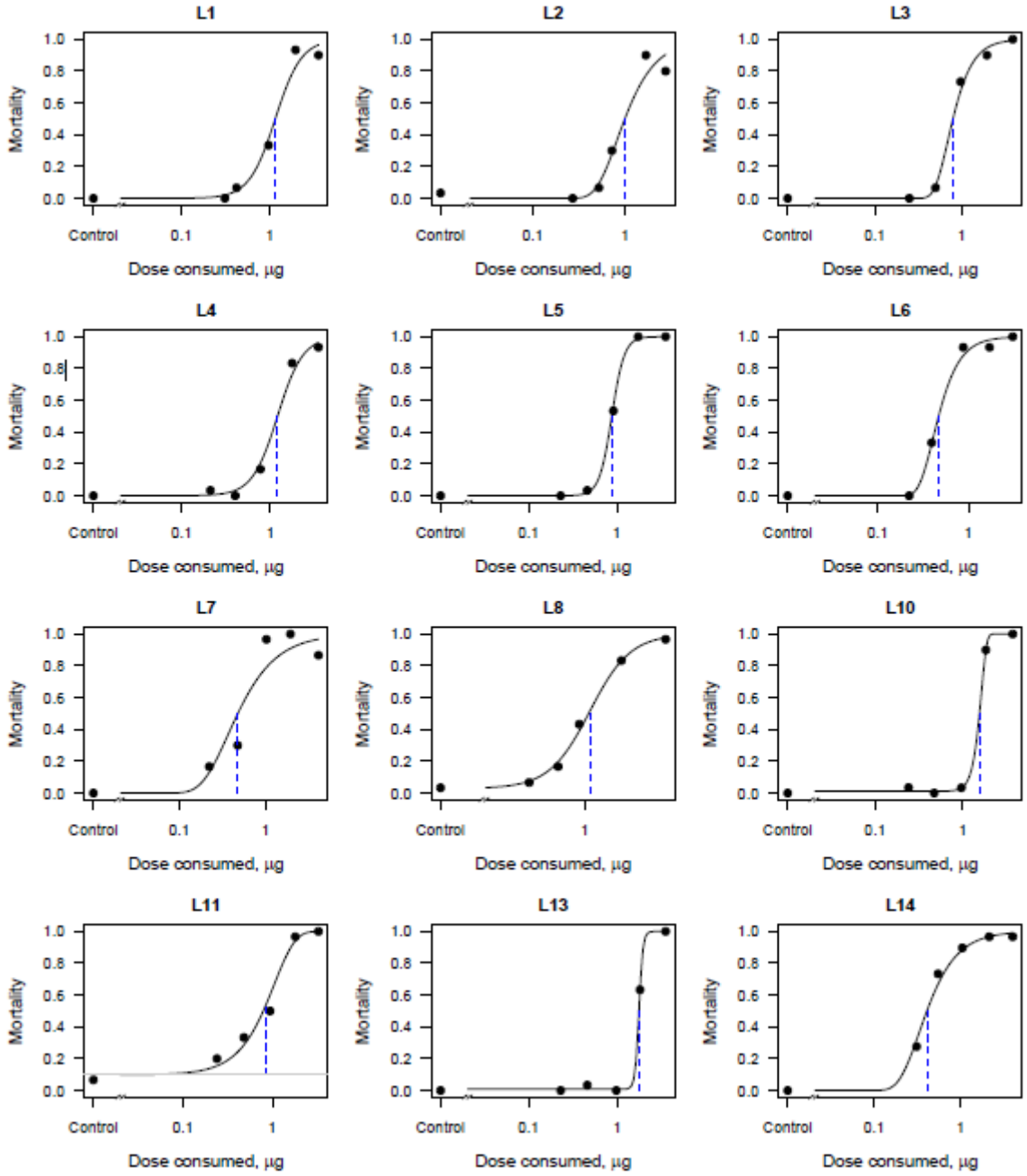

Figure 15: Dose-response models fitted to the oral test data obtained at 96 h after exposure (based on the average actual consumed doses). The x-axis is on the log scale. The blue dashed lines denote LD_{50}. Note that in cases when mortality in control bees was estimated to be non-zero, these blue lines start from a non-zero level shown as a light grey horizontal line.

Table 22: Summary of mean mortality generated in the oral test by laboratory 1 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
0.25	0	0	0	0
0.5	1	2	2	2
1	10	10	10	10
2	27	27	27	28
4	27	27	27	27

Table 23: Summary of mean mortality generated in the oral test by laboratory 2 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	1	1
0.25	0	0	0	0
0.5	1	2	2	2
1	8	9	9	9
2	27	27	27	27
4	24	24	24	24

Table 24: Summary of mean mortality generated in the oral test by laboratory 3 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
0.25	0	0	0	0
0.5	0	2	2	2
1	20	22	22	22
2	26	26	27	27
4	30	30	30	30

Table 25: Summary of mean mortality generated in the oral test by laboratory 4 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	er of dead bees]			
untreated control	0	0	0	0
0.25	1	1	1	1
0.5	0	0	0	0
1	4	5	5	5
2	25	25	25	25
4	28	28	28	28

Table 26: Summary of mean mortality generated in the oral test by laboratory 5 at each time point

treatment	**24h**	**48h**	**72h**	**96h**
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
0.25	0	0	0	0
0.5	1	1	1	1
1	14	15	16	16
2	30	30	30	30
4	30	30	30	30

Table 27: Summary of mean mortality generated in the oral test by laboratory 6 at each time point

treatment	**24h**	**48h**	**72h**	**96h**
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
0.25	0	0	0	0
0.5	9	10	10	10
1	28	28	28	28
2	28	28	28	28
4	30	30	30	30

Table 28: Summary of mean mortality generated in the oral test by laboratory 7 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
0.25	2	3	3	5
0.5	7	9	9	9
1	29	29	29	29
2	29	30	30	30
4	21	26	26	26

Table 29: Summary of mean mortality generated in the oral test by laboratory 8 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	1
0.25	1	1	1	2
0.5	4	4	5	5
1	13	13	13	13
2	25	25	25	25
4	29	29	29	29

Table 30: Summary of mean mortality generated in the oral test by laboratory 10 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
0.25	1	1	1	1
0.5	0	0	0	0
1	1	1	1	1
2	27	27	27	27
4	30	30	30	30

Table 31: Summary of mean mortality generated in the oral test by laboratory 11 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	2
0.25	0	2	5	6
0.5	1	1	4	4
1	8	12	14	15
2	22	29	29	29
4	30	30	30	30

Table 32: Summary of mean mortality generated in the oral test by laboratory 13 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
0.25	0	0	0	0
0.5	1	1	1	1
1	0	0	0	0
2	18	19	19	19
4	30	30	30	30

Table 33: Summary of mean mortality generated in the oral test by laboratory 14 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
0.25*	4	7	8	8
0.5	66,7	73,3	73,3	73,3
1	89,7	89,7	89,7	89,7
2	96,7	96,7	96,7	96,7
4	96,7	96,7	96,7	96,7

* 29 instead of 30 bumblebees were used in this treatment

2.1.2 Summary of data from laboratories in the oral test excluded due to not measuring the actual food uptake (L9; L12; L15)

Table 34: Cumulative mortality in [%] in the control and different test item treatments after 96 h showing also the data from the excluded laboratories L9; L12 and L15

Treatment	L1	L2	L3	L4	L5	L6	L7	L8	L9*	L10	L11	L12*	L13	L14	L15*	Mean
Control treatment																
0	0	3.3	0	0	0	0	0	3.3	3.3	0	6.7	0	0	0	5	1.4
Test Item treatments: Dimethoate (EC 400)																
0.25	0	0	0	3.3	0	0	16.7	6.7	3.3	3.4	20	0	0	27.6	5	5.7
0.5	6.7	6.7	6.7	0	3.3	33.3	30	16.7	16.7	0	33.3	0	3.3	73.3	7.5	15.8
1	33.3	30	73.3	16.7	53.3	93.3	96.7	43.3	53.3	3.3	50	3.3	0	89.7	17.5	43.8
2	93.3	90	90	83.3	100	93.3	100	83.3	90	90	96.7	73.3	63.3	96.7	35	85.2
4	90	80	100	93.3	100	100	86.7	96.7	100	100	100	100	100	96.7	80	94.9
LD_{50} values																
LD_{50} [µg/BB]	1.2	1.0	0.8	1.2	0.9	0.5	0.5	1.1	1.0	1.6	0.9	1.7	1.8	0.4	2.6	1.1

* Laboratories excluded in Table 2 and the results section due to not weighing the syringes.

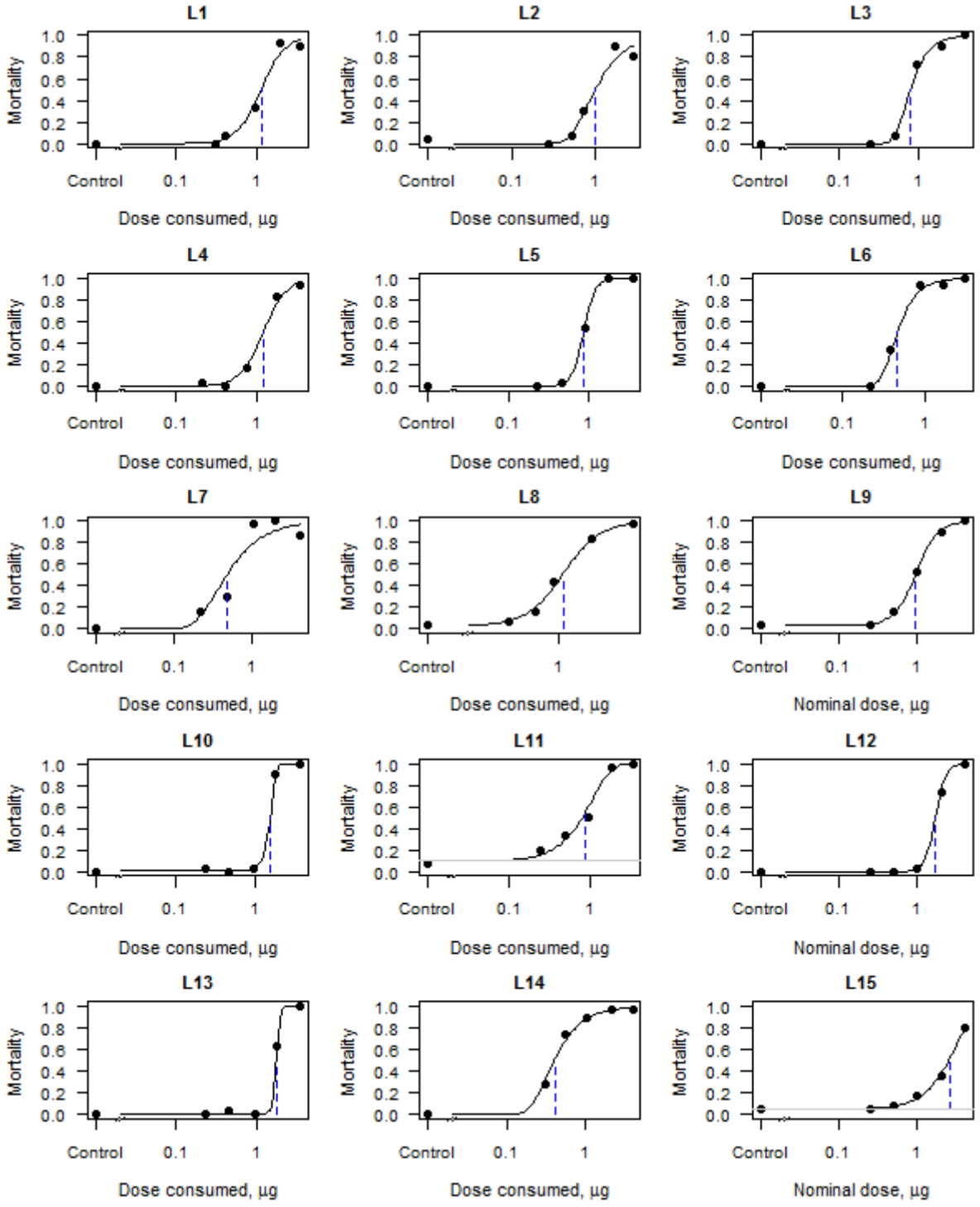

Figure 16: Dose-response models fitted to the oral test data obtained at 96 h after exposure (based on the actual consumed doses) showing also the data from the excluded laboratories L9, L12 and L15 (based on nominal doses). The x-axis is on the log scale. The blue dashed lines denote LD_{50}. Note that in cases when mortality in control bees was estimated to be non-zero, these blue lines start from a non-zero level shown as a light grey horizontal line.

Table 35: Summary of mean mortality generated in the oral test by laboratory 9 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	1	1	1	1
0.25	0	0	1	1
0.5	1	4	5	5
1	16	16	16	16
2	24	24	24	27
4	30	30	30	30

Table 36 Summary of mean mortality generated in the oral test by laboratory 12 at each time point

treatment	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	0	0	0	0
0.25	0	0	0	0
0.5	0	0	0	0
1	1	1	1	1
2	22	22	22	22
4	30	30	30	30

Table 37: Summary of mean mortality generated in the oral test by laboratory 15 at each time point

treatment*	24h	48h	72h	96h
[µg / bumblebee]	[number of dead bees]			
untreated control	1	2	2	2
0.25	2	2	2	2
0.5	2	2	3	3
1	7	7	7	7
2	14	14	14	14
4	32	32	32	32

* 40 instead of 30 bumblebees were used in all treatments

Summary of data from laboratories excluded in the oral test due to high mortalities across all dose levels of Dimethoate (Laboratory A and Laboratory B)

72. Oral test data were submitted by a total of 17 laboratories. However, a preliminary data analysis showed that no sensible dose-response models could be fitted to the data from two of these laboratories due to a similar high mortality even at low doses of Dimethoate (Figure 17). Therefore, the oral test data from these two laboratories were excluded from further consideration.

73. Control mortality (0 % and 3.3 %) in these two experiments is within the range of all other experiments. The observed mortality (Figure 17) in the test item doses does not appear to be method-related, therefore we consider the presented approach to be suitable for toxicity testing in bumblebees.

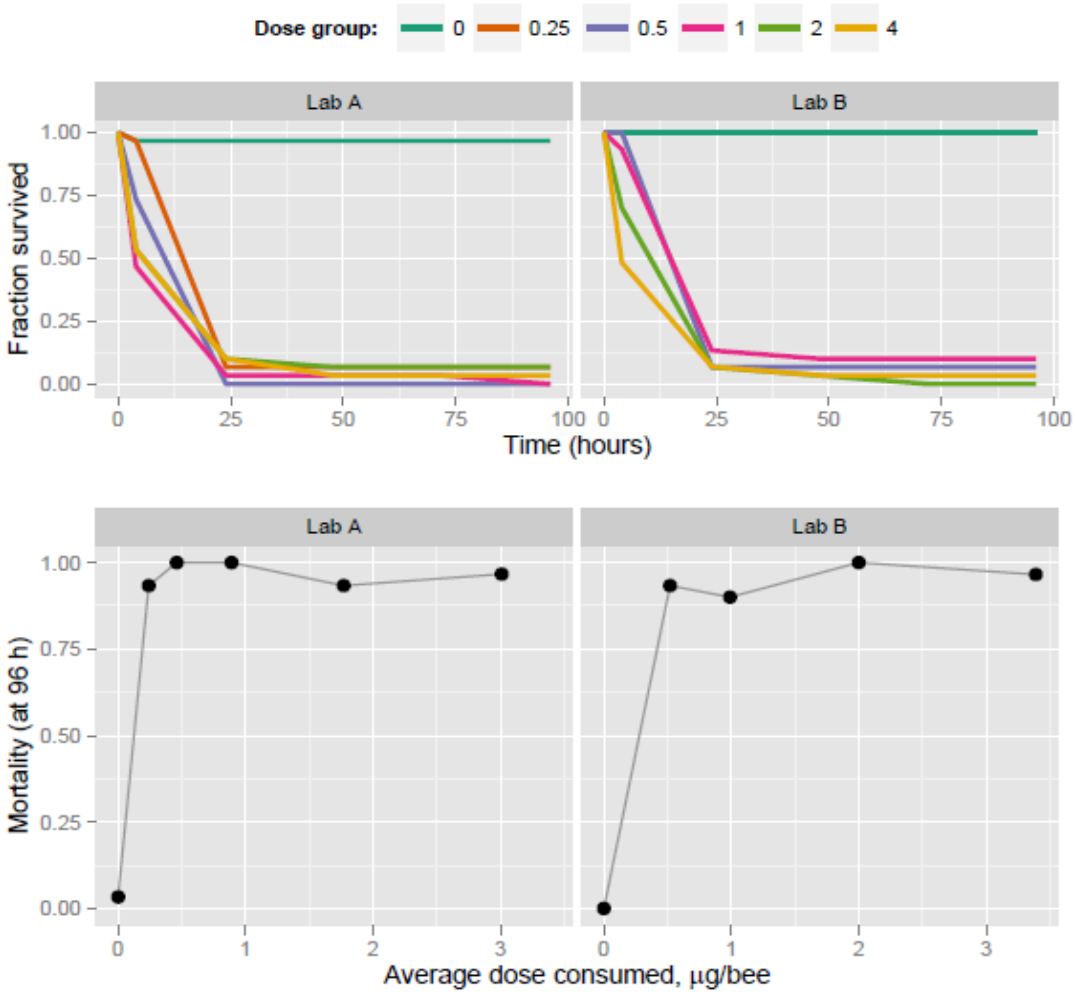

Figure 17: Results of the oral tests conducted on Bombus impatiens by two different laboratories (denoted here as "Lab A" and "Lab B"). Upper row: survival of the bees at different times after exposure. Bottom row: survival rate as a function of dose at 96 h after exposure. The undifferentiated toxic effect recorded in the Dimethoate-treated bumblebees made fitting any reasonable dose-response models impossible.