Methods in Ecology and Evolution

British Ecological Society

Methods in Ecology and Evolution doi: 10.1111/j.2041-210X.2011.00099.x

Honey bee risk assessment: new approaches for *in vitro* larvae rearing and data analyses

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Summary

1. To sustain the vital ecosystem service of pollination, new methodical developments are needed for research on the underlying factors of globally observed bee losses. In particular, robust laboratory methods for assessing adverse effects on honey bee brood are required. In addition, from a statistical point of view, the shared origin of test individuals must be considered when analysing ecotoxicological data.

2. To improve honey bee *in vitro* rearing, we adopted a nongrafting method to collect honey bee larvae without direct manipulation. Linear mixed effects model to evaluate LD_{50}, larvae survival and prepupae weights integrated the colony background of larvae as a random factor into the statistical analyses. The novel rearing approach and appropriate statistical tools for data analyses are illustrated in an *in vitro* case study on acute oral dimethoate toxicity.

3. We recommend our honey bee larvae collection approach for *in vitro* larvae-rearing applications, because of (i) a mere 3% background mortality upon the prepupae stage, (ii) a high quantitative capacity and (iii) because of robustness of performance which are great benefits for standardization.

4. The analyses indicate clear adverse effects of dimethoate by a significant survival reduction and prepupae weight reduction. For second instars, the acute 48-h LD_{50} was 1·67 μg dimethoate per larva.

5. We conclude that both our larvae collection method and the applied statistical approaches will help to improve the quality of environmental risk assessment studies on honey bees, to secure honey bee pollination and to sustain biodiversity.

Key-words: *Apis mellifera*, artificial comb, bioassay, colony collapse disorder, dimethoate, ecotoxicology, grafting, larvae collection, mixed effect model, pollination

Introduction

The worldwide losses of honey bee colonies have been raising genuine public concern. As global declines continue for many other social and solitary bees, the deterioration of insect-mediated pollination may critically affect agricultural and natural ecosystems (Fontaine *et al.* 2006; Potts *et al.* 2010). As a result, human food security is at stake because insect pollination is required for many kinds of seeds, fruits, vegetables and forage crops (Klein *et al.* 2007).

In recent years, researchers have reported numerous possible explanations for the phenomenon of disappearing honey bees, also known as colony collapse disorder (CCD) or honey bee depopulation syndrome (HBDS). However, despite a high concern, conclusive clarification of CCD has not yet been found (vanEngelsdorp *et al.* 2009). To address the multiple open questions on colony losses, the development of effective and practical honey bee risk assessment approaches is imperative. Advances are needed in the development of field, semi-field and laboratory standard testing methods. On the other hand, appropriate multi-factorial data analysis methods have to be applied to integrate different explanatory variables, such as the genetic origin of honey bee colonies, pathogenic pressures, landscape structure and exposure to environmental pollution or agricultural pesticides.

The health of honey bee brood is a crucial factor for colony survival. During the larval phase, environmental conditions play a formative role in the behaviour and longevity of bees (Becher *et al.* 2010). Feeding on pollen and nectar in the larval diet directly exposes larvae to the environment (Haydak 1970; Babendreier *et al.* 2004). Pollen or nectar containing pesticides may have detrimental consequences for the brood of a colony;

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therefore, laboratory methods for assessing adverse effects on larvae development are required.

In contrast to experiments in colonies (*in vivo*), which can be biased by many uncontrolled factors, the rearing of larvae in the laboratory (*in vitro*) is a highly attractive assay because of controlled laboratory conditions, reproducibility and the defined amounts of ingested test doses by larvae (Aupinel *et al.* 2007). Biologically relevant *in vitro* assessment endpoints such as the survival and weight of test individuals can be monitored in a straightforward manner. This methodology could develop into a routine standard environmental risk assessment bioassay. However, many honey bee laboratories still face multiple challenges (COLOSS, 2010), which shows the urgent need of further *in vitro* bioassay approaches. Many tests *in vitro* are hampered by occasional high mortality rates, a lack of standardization and repeatability, as shown by the variance between different European laboratories in a standardized dimethoate LD_{50} ring test (Aupinel *et al.* 2009). The causes of the observed variance may lie in practical experimenter skills, the season, genetic variation and larval age heterogeneity at grafting (Aupinel *et al.* 2009).

Within our study, we address many *in vitro* larvae-rearing challenges: an easy collection of tests larvae, the use of age-defined test larvae, a low larval mortality, the comparability between *in vitro*- and *in vivo*-developing larvae, standardization of protocols and the benefit of using up to date statistical applications. We introduce statistics that take into account that workers from one colony share the same environment and that they are all the progeny of a single mother queen. These facts have not yet been statistically implemented in ecotoxicological studies of honey bees. However, recent social insect studies correct for the multiple colony backgrounds among tested workers (Bocher *et al.* 2007; Koyama *et al.* 2007; Kasper *et al.* 2008; Muller & Korb 2008; Zuur *et al.* 2009; Castella, Christe & Chapuisat 2010).

The main goal of this study is to unite new approaches for *in vitro* larvae rearing. We present a novel artificial comb-based larvae collection method with the capacity to improve standardization between laboratories. We test the reliability and robustness of the new larvae collection approach. As the mechanical stress of traditional grafting is bypassed, the viability of collected individuals is optimized. The benefits of this method are illustrated in a case study, testing the reference insecticide dimethoate on honey bee larvae survival rates, weight and lethal dose values. The larvae collection method and the endpoint evaluation statistics will help to standardize *in vitro* rearing bioassays, to facilitate ecotoxicological studies on honey bees.

Materials and methods

A NEW LARVAE COLLECTION PROTOCOL

First instar larvae were collected using artificial combs of the commercially available system for queen breeding (Cupularve Nicotplast©, Maisod, France). In preparation for *in vitro* rearing trials, artificial combs were mounted onto wooden frames with honey comb. Each artificial comb (10 × 10 cm) is made of plastic and has 110 honey bee cell-sized holes (see pictures Appendix S1; Supporting Information). Crystal polystyrene plastic queen cups were placed over the cells at the backside and covered with a transparent lid. To allow bees to become familiarized with the combs, the frames were introduced into colonies 1 week preceding the experiments.

On 3 days (12th, 23rd and 25th of June 2009), queens were trapped on the artificial combs within their colonies by means of a plastic queen excluder lid. The queens of the test colonies were selected from six Upper Franconian apiaries to cover a variety of different *Apis mellifera carnica* genotypes. On day four (D4), eggs started to hatch in the cups (Table 1). At mean 92:59 h ± 1:50 SD after queen enclosure ($n = 13$), the cups with first instar larvae were collected from the colonies. Considering a 72-h development time of eggs (Bertholf 1925), the collected larvae had a mean chronological age of 10:29 h ± 0:55 SD and were at the biological age of being a first instar larvae.

To indicate the swiftness of this larvae collection procedure, at the 28th of June, it took two persons 45 min to collect 519 first instar larvae (mean 87 larvae per comb). At a speed of 12 harvested first instar larvae per minute, one person collected and replaced cells on the combs, while the other handled the combs at the colonies.

Collected cups with larvae were placed in 48-well culture plates with a humidifying piece of dental cotton roll at the bottom of each well, wetted with a 0·4% methylbenzethonium chloride and 15·5% glycerol/H_2O solution (Aupinel *et al.* 2005, 2007). During collection, plates with larvae were stored in a 35°C warmed polystyrene box for transport from the apiary to the laboratory.

To quantify the efficiency of our first instar larvae collection method, survival of 1060 nongrafted larvae was evaluated over the initial 24-h of *in vitro* rearing (see Table 1, D4–D5; Table 2, experiment 1a).

PROTOCOL FOR *IN VITRO* REARING

Honey bees were reared in the laboratory according to the protocol of Aupinel *et al.* (2005, 2007). The culture plates with larvae were kept in a hermetic plexiglass desiccator in an incubator at 35°C. Larvae were fed over D4 to D9 with a 10-, 10-, 20-, 30-, 40- and 50-μL semi-artificial diet, respectively. This diet consisted of 50% royal jelly (Le Rucher du Buzard certified organic apiary, Sospel, France) mixed with a 50% aqueous solution. The yeast extract/glucose/fructose proportion in the aqueous solutions was, respectively, 2/12/12% at D4 and D5; 3/15/15% at D6; and 4/18/18% at D7, D8 and D9. During larval development, rela-

Table 1. Developmental stages of honey bee individuals upon hatching (after Bertholf 1925), with key days for *in vitro* larvae-rearing experiments marked in grey. Key days are D4 for collection of first instar larvae, D5 for dimethoate treatment applications, D11 for weighing prepupae (PP) and as survival endpoint for larvae development and D21 as checkpoint on the quality of hatched individuals.

D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21
egg			L1	L2	L3	L4	L5		PP		pupae									

Table 2. Overview of performed experiments with the number of larvae and colonies used to evaluate the measured parameters survival, weight and LD_{50}.

	Experiment	*n* (larvae)	Colonies	Survival	Weight	LD_{50}
1a	Non-grafting larvae	1060	7	D4–D5		
1b	Larvae rearing	106	7	D5–D11	D11	
1c	*In vivo*/hive control	18	3		D11	
1d	Weighting mortality	103	7	D11–D21	D21	
2	Dimethoate gradient	99	7	D5–D11	D11	D5–D7

tive humidity in the incubator was kept at 96% using a saturated solution of K_2SO_4. Further development upon hatching took place in 80% humidity, maintained using a saturated solution of NaCl. Climatic conditions were monitored with data loggers (MSR electronics, Henggart, Switzerland).

PERFORMANCE OF *IN VITRO* REARING

Successful larvae development upon the prepupae phase indicates a high performance of the *in vitro* rearing method. Therefore, the D4–D5 survival measurements of 1060 first instar larvae were followed by subsequent D5–D11 survival evaluation of 106 *in vitro*-reared larvae (Table 2, experiment 1b).

At D11, weight data (±1 mg) were collected by transplanting prepupae with soft metal tweezers into a new plastic queen cup on a microbalance, to enable a sound prepupae weight comparison between treatments. Additionally, we show an artificial comb application to compare the weights of *in vitro*-reared prepupae (laboratory) with *in vivo* prepupae which develop in parallel on the artificial combs inside honey bee colonies (Table 2, experiment 1c).

A subsequent survival comparison over D11–D21 was used to measure mortality effects of the manipulation of prepupae for weighing (Table 2, experiment 1d). Therefore, the survival of unmanipulated prepupae was compared with the survival of transplanted prepupae.

The test individuals were daily monitored under a stereomicroscope to verify health: moribund and dead test individuals, recognized by occasional black or white sub-dermal necrotic stains or a visible loss of turgor, were removed. Additional quality checks on *in vitro* hatching honey bees (D21) were performed by measuring weight and checking the inter-caste characteristics of workers by a basitarsal pollen comb inspection, as described by Allsopp, Calis & Boot (2003).

DIMETHOATE TOXICITY TEST DESIGN

For ecotoxicological endpoint evaluation for an *in vitro* honey bee bioassay, a case study on dimethoate was performed. The acute oral toxicity of a dimethoate concentration gradient was tested on 99 *in vitro*-reared larvae. The test doses were 0/0·2/0·8/3·2/12·8 μg $larva^{-1}$ (Aupinel *et al.* 2007, 2009) on $n = 20 \pm 1$ larva per dose. The dimethoate was obtained from Fluka Chemie, Switzerland. In contrast to Aupinel *et al.* (2007, 2009), we treated the second instar larvae (D5) by feeding dimethoate mixed in 10-μL artificial diet. The test was split over two experimental periods: trial I starting on June 23rd and trial II starting on June 25th. Covering all treatment levels, larvae from seven colonies were included.

The three evaluated toxicity endpoints were (i) the survival of larvae to indicate lethal effects, (ii) the weight of prepupae for indicating possible sub-lethal effects and (iii) an 48-h LD_{50} acute oral toxicity value of dimethoate for the exposed second instar larvae (see also: Statistical analyses).

STATISTICAL ANALYSES

One objective of this study is to apply the shared origin of honey bee workers in the analysis of ecotoxicological data sets. Different packages of the open source statistic software R version 2·11·1 were used. (R Development Core Team 2010). Colony identity was always included as random factor in the models to compensate for the identical background of larvae. The dimethoate treatment gradient with five concentration levels was the key predictor. Trial was a fixed factor with two levels (starting on the 23rd or 25th of June) which was always tested, but was removed from the models when direct *P*-values or likelihood ratio tests did not indicate it as a significant explanatory variable (Zuur *et al.* 2009).

Survival analysis

The larval survival was analysed with a Cox proportional hazards regression model (Fox 2002) using the R packages survival and survnet (Ripley, Harris & Tarassenko 2004; Therneau 2009). The *P*-values from the Cox model summary, which indicate differences between the control and the individual dimethoate levels, were corrected with the Holm–Bonferroni procedure (Holm 1979) to provide a multicomparison correction.

Prepupae weight analysis

To investigate whether the endpoint prepupae weight was affected by dimethoate, we applied a linear mixed effects model using the package lme4 (Bates & Maechler 2010). The treatment analysis was performed on the dimethoate gradient as covariate predictor variable. The doses (+0·001 μg) were $\log_{10}$ transformed to linearize the exponentially progressing gradient. The prepupae weight model was checked visually on normality of the residuals by normal probability plots, and we assured the homogeneity of variances and goodness of fit of the model by plotting residuals vs. fitted values (Faraway 2006). The program R does not directly provide *P*-values for mixed effect models with the package lme4, so we extracted them using likelihood ratio tests (LRT) based on the changes in the deviance when an explanatory variable was dropped from the full model (Rödel *et al.* 2010).

LD_{50} analysis

As acute oral toxicity endpoints, LD_{50} values were calculated from survival data of a 48-h dimethoate exposure to second instar larvae. A generalized linear mixed effects model (glmer) was fitted with colony identity included as a random factor. This approach is compared with a standard logistic regression analysis, using a generalized linear model fit (glm). Treatment entered both models as covariate with $\log_{10}$ transformed dimethoate doses (+0·001 μg), and for the survival data (dead or alive), the family function 'binomial' with the link function 'logit' was used.

Lethal dose values were calculated using the intercept (a) and treatment parameter estimate (b) from the models. At 50% mortality, the

log10 dose estimate is -(a/b) and the $LD_{50} = 10^{-(a/b)}$. The 95% confidence intervals of the mixed model LD_{50} value were calculated with Fieller's method (Finney 1971; Niu, Johnson & Berenbaum 2010).

Results

LARVAL COLLECTION AND *IN VITRO* REARING PERFORMANCE

The novel approach for larvae collection performed well, both quantitatively and qualitatively, considering that 1043 of 1060 first instar larvae (98·4%) survived the first 24 h of *in vitro* rearing (D4–D5). The robustness of our method is repetitively shown over the three collection days, by a survival rate of 100% ($n = 64$), 97·6% ($n = 531$) and 99·1% ($n = 465$), respectively.

Subsequently, of the 106 larvae of experiment 1b, 99·1% reached the fifth instar stage (D5–D9) and 97·2% the prepupae stage (D5–D11), indicating successful rearing performance over the larval stages.

The mean weight of *in vitro*-reared prepupae was 141·4 mg ± 1·4 SE ($n = 20$). In contrast, *in vivo* prepupae reared on artificial combs in colonies were significantly heavier (mean 165·2 mg ± 2·84 SE; $n = 18$) than *in vitro* prepupae (*t*-test: $t = -7{\cdot}758$; d.f. $= 36$, $P < 0{\cdot}001$).

The emergence rates of prepupae (D11–D21) were significantly affected by the manipulation of prepupae for weighing (Fig. 1). Unmanipulated prepupae showed an emergence rate of 81%, whereas emergence rate of weighed prepupae was significantly lower at 30% (Cox proportional hazards regression: $X^2 = 33{\cdot}27$; d.f. $= 1$, $P < 0{\cdot}001$).

Hatched *in vitro* adults were all morphological workers having typical worker pollen combs, with a mean weight of 126·1 mg ± 5·0 SE ($n = 10$).

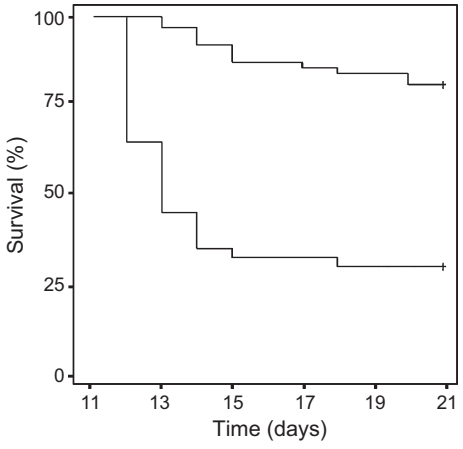

Fig. 1. Influence of prepupae weighing on the survival of *in vitro* developing pupae. Shown is postweighing survival (> day 11) upon the hatching of bees at day 21 ($n = 63$ unmanipulated vs. 40 manipulated larvae).

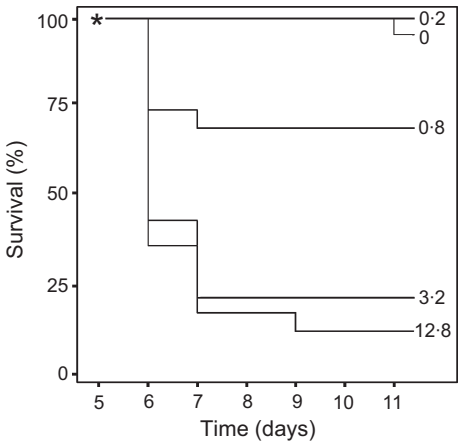

Fig. 2. Effects of dimethoate on survival of *in vitro*-reared honey bee larvae. Survival characteristics of untreated and dimethoate-treated larvae are indicated by a Cox proportional hazards regression model. The survival dynamics from the treatment day (D5) up to the prepupae phase (D11) are shown. The units 0, 0·2, 0·8, 3·2 and 12·8 indicate the dimethoate treatment doses in µg per larva as applied in the diet of second instar larvae (D5).

ECOTOXICOLOGICAL ANALYSES

The dimethoate treatment gradient clearly affected the endpoint survival over the larval phase (Fig. 2, Table 3 & 4). The post hoc comparisons among treatment levels showed that at 0·2 µg dimethoate, the survival among larvae was identical to the control larvae. A decrease in survival over the dimethoate gradient started as trend at the level of 0·8 µg $larva^{-1}$, and at both the doses 3·2 µg and 12·8 µg dimethoate, a significantly higher mortality rate compared to controls was found (Table 3, Fig. 2).

A significant decrease in mean prepupae weight occurred with increasing dimethoate concentration (Table 4). Between the two experimental trials, starting on 23 or 25 June, a difference in mean prepupae weight was found. The prepupae were mean 2·9 ± 2·0% SD ($n = 4$ doses) significantly heavier in the second trial (Table 4).

Acute larvae mortality by dimethoate exposure was indicated, as treatment was a significant factor in the generalized linear mixed effect model (Table 4). The mixed model approach showed a dimethoate 48 h LD_{50} of 1·67 µg $larva^{-1}$ ($n = 99$) with 95% confidence intervals of 0·84 and 3·30 µg $larva^{-1}$ (Fig. 4). The standard regression method resulted in a LD_{50} of 1·69 µg dimethoate (95% confidence interval of 1·03–5·48 µg $larva^{-1}$).

Discussion

LARVAL COLLECTION AND *IN VITRO* REARING PERFORMANCE

Improving methods to determine the underlying reasons for recent honey bee colony declines is imperative. Here, we present effective approaches for larvae collection and data analyses, which can help facilitate *in vitro* rearing

Table 3. Cox proportional hazards regression models with the colony background of test larvae included as random factor, to compare survival dynamics of *in vitro*-reared larvae between control and dimethoate treatments.

Dimethoate per larvae Level comparisons	d.f.	X^2	Bonferroni-Holm corrected α	P-value[1]
0 µg vs.0.2 µg	1	0.00	0.05	1.00 ns
0 µg vs.0.8 µg	1	4.55	0.025	0.034 ns
0 µg vs.3.2 µg	1	13.12	0.017	< 0.001***
0 µg vs.12.8 µg	1	14.72	0.0125	< 0.001***

[1] P-values are given, with indicators for high significance (***) and no significance (ns).

Table 4. Dimethoate test overview on models and results on the tested endpoints survival, weight and the 48-h lethal dose value.

Endpoint	Model	Effects[a]	d.f.	X^2	P-value[b]
Survival (D5–D11)	Cox proportional hazards regression model	Fixed factor(treatment)	4	67.4	< 0.001***
		Fixed factor(trial)	1	0.2	0.64 ns
Prepupae (Weight D11)	Linear mixed effects model (with LRT[1])	Fixed(treatment)	1	14.2	< 0.001***
		Fixed factor(trial)	1	4.3	0.038*
48 h LD_{50} (D5–D7)	Generalized linear mixed effects model	Fixed(treatment)	1	67.4	< 0.001***
		Fixed factor(trial)	1	1.7	0.19 ns

[a]Effects for the predictor variables are derived from the model summaries or from likelihood ratio tests (LRT).
[b] P-values are given, with indicators for high significance (***) and no significance (ns).

methodology and benefit the accuracy of honey bee risk assessment studies. We clearly show that larval collection with artificial combs generates very low mortalities and is practical, quick and easy. The new nongrafting approach helps to improve *in vitro* rearing techniques for fundamental and applied honey bee research. In addition, the use of the artificial comb system enabled to include a number of further refinements, which result in a higher standardization level of honey bee brood tests.

Of 1060 collected first instar larvae, 98·4% survived the first critical 24 h of *in vitro* development. Because eggs and young larvae are physically vulnerable, it is to be expected that minimizing manipulation contributes to the survival of larvae. Where traditional grafting with brushes, needles or feathers may cause mechanical stress and mortality among larvae, the artificial comb-based method enables first instar larvae collection without grafting larvae, by letting queens lay eggs directly into the test vessels for *in vitro* experiments. Depending on experience, the success of grafting is never guaranteed and often many eggs or larvae do not develop. Wegener, Al–Kahtani, & Bienefeld (2009) reported 75% of grafted eggs being retrieved as viable larvae. Evans, Boncristiani & Chen (2010) noted only 43% survival of needle-grafted eggs. Often, data on the collection success of test individuals remain unmentioned in papers on *in vitro* rearing (Aupinel *et al.* 2005, 2007, 2009; Behrens *et al.* 2007; Brodschneider, Riessberger-Gallé & Crailsheim 2009; Jensen, Pedersen & Eilenberg 2009). It is common practice to replace unviable larvae by spare larvae at the moment of treatment to compensate for grafting mortality. By bypassing the grafting procedure, this methodological shortcoming was solved. In the absence of grafting mortality, experimental time upon the prepupae phase is gained and thereby creating the opportunity to test the very sensitive first instar stage. In contrast to other artificial comb systems (Omholt *et al.* 1995; Aase *et al.* 2005), only the adopted Cupularve system allows the pragmatic collection of larvae within plastic cups, which are at the same time the perfect test vessels for *in vitro* rearing experiments.

As shown, 99% of *in vitro*-fed larvae reached the fifth instar stage and 97% the prepupae phase. For a bioassay, this high survival rate is optimal to sensitively test acute or chronic mortality effects on developing larvae. Compared to 10% control larvae mortality in the chronic dimethoate test by Aupinel *et al.* (2007), our 2·8% background mortality upon the prepupae phase is a definite advancement.

In terms of standardization, our approach without direct human interference would guarantee a constant quality of *in vitro* larvae. As larvae collection is the primary step for *in vitro* rearing, the use of this robust approach would minimize experimenter-generated variance. This is because of the dependency of traditional grafting on the experience of the human manipulator. Potentially, the comparability between laboratories is greatly enhanced when studies adopt our approach to achieve general low background mortality. An essential challenge for honey bee laboratories is to overcome high mortality among controls (Janke *et al.* 2010). The validity criteria that mortality should not exceed 15% at the end of acute 48-h toxicity test (Aupinel *et al.* 2009) are by means of our methodology substantially secured. Thus, the results support the recommendation of the comb collection method to other laboratories that perform *in vitro* exposure bioassays.

Worth stipulating is the general practicability of using the nongrafting queen-rearing system. It is a great advantage that

grafting with needles is no longer necessary. Collection of larvae can be performed without much experience because the method is straightforward. Also, the quantity of comb-collected larvae indicates the potential for performing elaborate experiments. The collected numbers of first instar larvae should amply suffice for extensive experimental designs. In 45 min, including hive handling time, over 500 larvae were collected. Evans, Boncristiani & Chen (2010) describe grafting 220 eggs in 30 min, which shows that our technique is comparable to the collection speed of experienced grafters. Compared to the procedure of Evans, Boncristiani & Chen (2010), basically gathering many eggs by hitting honey bee combs on the table, our mass collection method is considerably more refined.

We stipulate that quickness of collection is important, because it is likely a relevant factor to rearing successes. The larvae are less exposed to outside-hive conditions, which can be unfavourable because of dehydration, low temperatures, fungal contamination and UV light.

A common rearing problem for the northern European bee season is that early and late in the season, collection of viable larvae is inconvenienced because of a low amount of eggs, unfavourable grafting conditions and low willingness of bees to nurse young larvae (Aupinel *et al*. 2005, 2009). The use of artificial combs might help by making *in vitro* rearing less season dependent. Because queens are enclosed on the small comb area, searching time for collectable larvae is reduced. The swiftness of cup collection lessens damage to the larvae by unfavourable conditions. Optional additional sugar/protein feeding could stimulate queens to lay eggs and workers to nurse larvae on the combs.

Aupinel *et al*. (2005, 2007) described the preference to confining queens for 30 h for harvesting plentiful larvae. It is hypothesized that heterogeneity of larvae ages and a possible age-depending susceptibility towards insecticides can explain differences in test outcomes (Aupinel *et al*. 2009). Accordingly, we promote a higher age standardization for *in vitro* test larvae, preferably an instar stage–related age range (Table 1). By timing the harvest moment, the age range of test individuals can be controlled, limiting the maximum age, securing collection of first instar larvae. For smaller age variances, shorter queen enclosure time windows can be applied. For example, when queens are confined for 10 h and larvae are subsequently collected after 87 h, all larvae will be in the chronological age range of 5–15 h. For queen enclosure time manipulation, the artificial comb is a practical tool. The queens in the experiments did not show reluctance in laying eggs, because all artificial comb cells were filled with larvae or eggs at the harvest moment (see graph in Appendix S2; Supporting information). Finally, the use of young queens is recommended because they have a high egg laying capacity.

Considering the low variance in prepupae weight data, the endpoint prepupae weight is exact and ideal for evaluating toxic effects on larvae. However, we like to point out that there is a clear mortality effect because of the manipulation of the prepupae (Fig. 1). Obviously, this developmental stage is extremely sensitive and the transplantation into a new cup causes lethal mechanical stress. This implicates that direct manipulation of prepupae is unrecommended for prolonged studies until hatchment. For studies upon emergence, the hatching weight is the appropriate assessment endpoint. Alternatively, a weight assessment endpoint of lower quality could be achieved by a method given by Aupinel *et al*. (2005), measuring prepupae within their cells. However, this causes weight anomalies because of food residues, moulting skins and defecation products that are simultaneously weighed. To our experience, these residues are also breeding grounds for fungi. At the cost of a mortality effect, a cell transplantation can still benefit methodology, because the weight data are accurate and the chance of fungal infections is actively suppressed.

Prepupae reared in the laboratory were 3·4% more heavy in weight compared to in hive individuals as described by Wedenig, Riessberger-Gallé & Crailsheim (2003). With a mean weight of 126 mg, hatched laboratory bees were 8% heavier compared to hatching hive bees, as reported by Bowen-Walker & Gunn (2001). Such differences can be explained by distinctive weight gain dynamics between *in vivo* and *in vitro* developing larvae, as described by Riessberger-Gallé, Vollmann & Crailsheim (2008).

Riessberger-Gallé, Vollmann & Crailsheim (2008) and Brodschneider, Riessberger-Gallé & Crailsheim (2009) performed studies focusing on the quality of *in vitro*-reared bees. *In vitro* honey bee risk assessments will be strengthened when test results are consolidated by proof that the development of test individuals was fully normal. We introduced with test 1c (Table 2), the innovative methodological control by artificial combs for in hive larval development, in parallel to laboratory *in vitro* developing larvae. The preliminary results showed that test individuals from the same batch, simultaneously developing *in vitro* and *in vivo*, can be compared.

Considering that 97% of *in vitro* control larvae reached the prepupae phase, that the weight of prepupae and hatched honey bees was bordering normality and that hatched worker bees were lively and had no inter-caste characteristics, we conclude that the presented method of *in vitro* rearing is adequate.

ECOTOXICOLOGICAL ANALYSES

In the dimethoate case study of *in vitro*-reared larvae, mixed models were successfully applied for each toxicity endpoint: survival over the larval phase, prepupae weight and a LD_{50} value (Table 4). By including colony background as a random factor, all models allowed statements for colonies in general, at the cost of only one parameter for colony identity (Zuur *et al*. 2009). This is in contrast to the general approach in honey bee ecotoxicology to assess colonies individually. In comparison, an analysis with colony background as a fixed factor would have the disadvantage of coming at the cost of six degrees of freedom (Zuur *et al*. 2009). It would also have the model handicap of not holding for colonies in general, but only for each colony individual (Zuur *et al*. 2009). For this reason, as long as individual colony responses are not the focus of a project, random intercept models are to be preferred.

From a biological point of view, the colony is for social insects the principle level of reproduction, survival and homeo-

stasis (Hölldobler & Wilson 2009). In addition, from a statistical perspective, honey bee worker data are considered nested because multiple observations are taken from the same colony (Zuur *et al*. 2009). Test individuals from the same colony share (i) the same environment (stressors on test larvae within colonies: bacterial/fungal/viral/chemical/nutritional/temperature) and they are related to each other (ii). Without considering tested colony mates as groups, models would fail to take into account a fundamental assumption of standard statistical models, the independence of errors (Crawley 2007).

Survival over the larvae phase was highly affected by dimethoate application in the case study (Table 3). Aupinel *et al*. (2007) found 5 days post-treatment, 40% and 85% prepupal mortality for the treatments 0·8 and 12·8 μg dimethoate, respectively. We observed 6 days post-treatment, for the same treatment doses a mortality rate in the same range; 32% and 89%, respectively (Fig. 2). As indicated by post hoc comparisons, the endpoint survival (from D5 to D11) is a clear and strong indicator of adverse effects to developing larvae. The Cox model uses chi-square statistics, which has limits to statistically discriminate differences when sample sizes are low. The tested sample size of 20 individuals per treatment level was at the boundary of indicating a possible treatment effect at 0·8 μg larva^{-1}. For this level in particular, a significant statistical discrimination was lost because of the sequential Holm–Bonferroni correction (Table 3). To optimize the statistical discrimination in similar test designs, the number of levels can be reduced and larvae sample sizes increased.

The linear mixed effects analyses indicated a significant reduction in prepupae weight over the dimethoate concentration gradient (Fig. 3). Relative low weight of a prepupae is likely to be caused by low food uptake because of intoxication. Heterogeneity in age is unlikely a variable of influence on

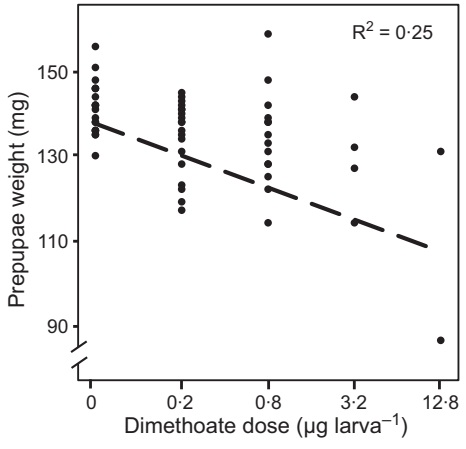

Fig. 3. Effects of dimethoate treatments on prepupae weights of *in vitro*-reared honey bee larvae. Prepupae weight sample sizes are, respectively, 20, 20, 13, 4 and 2 over the dimethoate gradient 0/0·2/0·8/3·2/12·8 μg larva^{-1}, as applied in the diet of second instar larvae. The regression line over the log transformed dimethoate dose gradient describes the significant trend for lower weight of prepupae at higher treatment doses (R^2 = 0·253; at X^2 = 14·2 with d.f. = 1 and P < 0·001, with the colony identities considered as a random factor).

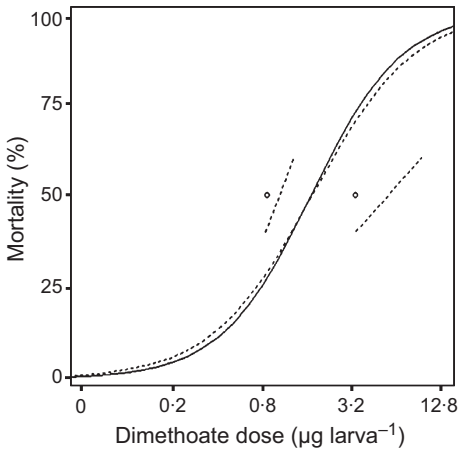

Fig. 4. Dose–response curves on mortality of *in vitro*-reared honey bee larvae (n = 99), 48 h after dimethoate exposure. The x-axis shows the exponential dimethoate dose gradient (0/0·2/0·8/3·2/12·8 μg larva^{-1}) with spacing factor 4. The y-axis shows the probability of mortality. Linear regression model fits were performed on mortality (logit transformed) and dimethoate dose (+0·001 μg and log10 transformed) to extract LD_{50} values. The dashed dose–response curve shows the standard regression approach (glm) with the 95% confidence interval (dashed lines). The continuous dose–response curve shows the mixed model approach (glmer) in which colony is included as random factor. The mixed model confidence interval is indicated by the two open points.

prepupal weights because normally larvae finish the administered diets to the full extent by D10 and as no longer food is consumed, their weight is stable by D11. Thus, a developmental heterogeneity is evened out as younger larvae catch up on the older larvae in reaching their maximum weight as prepupae. Trial was however a significant predictor variable (Table 4). The 2·9% weight difference between the two experimental trials indicates that the methodology can be further standardized. We can recommend for example the use a multipipette to apply highly constant amounts of diet to larvae.

Our study provides a scientifically sound assessment of a standardized LD_{50} toxicity value. The generalized linear mixed effect model indicated a dimethoate LD_{50} value of 1·67 μg with a confidence interval between 0·84 and 3·30 μg larva^{-1}. This corresponds to the 1·93 μg larva^{-1} and a 1·0–3·0 μg larva^{-1} confidence interval as reported by Aupinel *et al*. 2007. It lies also perfectly in range with the 1·5–3·1 μg larva^{-1} documented for a ring test carried out in seven different international laboratories (Aupinel *et al*. 2009). Hence, these data indicate that the method of rearing larvae, as well as the statistical approach, allows for an accurate and reproducible toxicity test. Adult bees by Hardstone & Scott (2010) were reported to have a dimethoate LD_{50} value of 1·62 μg g^{-1} body weight, which relates to acute 24 h-LD_{50} values of mean 0·16 μg bee^{-1} for topical exposure and mean 0·18 μg bee^{-1} for oral exposure (Gough, McIndoe, & Lewis 1994). In comparison, our LD_{50} at 1·67 μg larva^{-1} is roughly 10 times higher, although the larvae are both topically and orally exposed.

The comparison of the standard method of linear regression (Aupinel *et al*. 2009) with our mixed model approach is visualized by the two dose–response curves in Fig. 4. The presented

approaches of LD_{50} calculation are not considered significantly different because the 95% confidence intervals of the standard (glm) and the mixed effect (glmer) calculation are overlapping. The implementation of colony background in the LD_{50} analysis of this case study did not have a pronounced effect on the LD_{50} value, nonetheless the calculation correctly implemented the dependency of larvae originating from the same colony.

Considering the dimethoate test design, a reserved stance towards individual colony LD_{50} values is recommended. Colony values must be considered with caution when larvae numbers throughout colonies are unbalanced or when experimental designs do not provide high sample sizes per treatment level. Regressions with low sample sizes are sensitive to chance effects, resulting in a variance between lethal doses which is not necessarily colony or treatment related. Stochastically, a mixed model LD_{50} is more robust because the sample size is high, while colony backgrounds are included as a random factor.

As shown by the case study, tailor-made analyses for a variety of *in vitro* toxicity test endpoints are possible. In general, the strength of such modelling statistics is the robustness and flexibility in dealing with e.g. pseudo replications, repeated measurements, abnormal distributions and imbalances within the data set (Crawley 2007). It is innovative for honey bee bioassays that single effects, but also multiple effects and also interactions, can be evaluated by testing multiple explanatory variables. For example, this is suitable when analysing the interplay of different pesticides in varying doses on honey bees. CCD research should likewise benefit by the possibility of simultaneously analysing several potential stressors such as pesticides, parasites and malnutrition. For ecotoxicology studies on honey bees, we state that the presented statistical approaches have a high potential.

The comprehensiveness of bioassays benefits by the inclusion of a high number of colonies and a broad range of backgrounds. This is in contrast with the widely observed limited number of test colonies in bioassays. As colony origin may cause variance in toxicity outcomes, low colony numbers in experiments could lead to possible under- or overestimation in toxicity assessments. Also to be considered is brood temperature-related susceptibility towards toxicants (Medrzycki *et al.* 2010) and differences in susceptibility towards stressors between lineages (Behrens *et al.* 2007; Jensen, Pedersen & Eilenberg 2009). Also, brood development characteristics may differ between colonies (Collins 2004). An experimental bioassay design should address existing variations in susceptibility. Therefore, it is best to include a multitude of colonies comprehending a wide range of genetic and phenotypic variation, as present in the honey bee population.

Conclusions

To counteract the worldwide bee losses, the development and standardization of effective risk assessment studies on honey bees are needed. Following the basic idea of minimizing contact with the larvae to optimize rearing success, a number of *in vitro* rearing challenges are solved. We state that the presented nongrafting approach, which to date is never applied for *in vitro* rearing, is highly efficient for larvae collection. It has high potential for standardization and method improvement of fundamental and applied *in vitro* rearing honey bee research. Contacting test individuals is harming them, as shown by the high mortality rate after weighing manipulation.

We presented in this study suitable statistical methods for ecotoxicological data analyses for honey bee studies. As shown, multiple colony test designs can be evaluated using several explanatory factors on biologically relevant *in vitro* test endpoints, such as survival and weight. We conclude that a multi-colony approach with adequate statistical implementation to correct for highly related individuals benefits honey bee risk assessment studies.

Acknowledgements

This research was funded by the German ministry for education and research (BMBF project 0315215E, Berlin). We kindly thank Martina Janke (LAVES Institut für Bienenkunde, Celle), Sjef van der Steen (Wageningen University and Research Centre), Jochen Pflugfelder (ALP, Liebefeld), Polina Bam (University of Guelph), Stefan Gross and Alexandra Kästner (University of Bayreuth) for their support.

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Received 2 September 2010; accepted 5 February 2011
Handling Editor: Elizabeth Horne

Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Comb pictures.

Appendix S2. Larvae collection graph.

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