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Secondary biomarkers of insecticide-induced stress of honey bee colonies and their relevance for overwintering strength

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ABSTRACT

The evaluation of pesticide side-effects on honeybees is hampered by a lack of colony-level bioassays that not only are sensitive to physiological changes, but also allow predictions about the consequences of exposure for longer-term colony productivity and survival. Here we measured 28 biometrical, biochemical and behavioural indicators in a field study with 63 colonies and 3 apiaries. Colonies were stressed in early summer by feeding them for five days with either the carbamate growth regulator fenoxycarb or the neurotoxic neonicotinoid imidacloprid, or left untreated. Candidate stress indicators were measured 8–64 days later. We determined which of the indicators were influenced by the treatments, and which could be used as predictors in regression analyses of overwintering strength. Among the indicators influenced by fenoxycarb were the amount of brood in colonies as well as the learning performance and 24 h-memory of bees, and the concentration of the brood food component 10HDA in head extracts. Imidacloprid significantly affected honey production, total number of bees and activity of the immune-related enzyme phenoloxidase in forager bee extracts. Indicators predictive of overwintering strength but unrelated to insecticide feeding included vitellogenin titer and glucose oxidase-activity in haemolymph/whole body-extracts of hive bees. Apart from variables that were themselves components of colony strength (numbers of bees/brood cells), the only indicator that was both influenced by an insecticide and predictive of overwintering strength was the concentration of 10HDA in worker bee heads. Our results show that physiological and biochemical bioassays can be used to study effects of insecticides at the colony level and assess the vitality of bee colonies. At the same time, most bioassays evaluated here appear of limited use for predicting pesticide effects on colony overwintering strength, because those that were sensitive to the insecticides were not identical with those that were predictive of colony overwintering. Our study therefore illustrates the difficulties involved in evaluating the economic/ecological significance of pesticide-induced stress in honey bee field studies.

1. Introduction

In most countries, pesticides whose intended use implies a possible exposure of honeybees (*Apis mellifera*) have to be tested for negative effects on this economically and ecologically important species in order to achieve homologation (see for example European Union regulation 1107/2009; US-OCSPP-guidelines 850-3020 to 850-340). In recent years, the adequacy of existing testing schemes for plant protection products on honeybees has been questioned, both because of risen public awareness and of new scientific results highlighting the extent and importance of sublethal and/or delayed effects (Abramson et al., 2004; Decourtye et al., 2004; Di Prisco et al., 2013; Dively et al., 2015; Rondeau et al., 2014). Efforts are therefore being made to improve them (EPA, 2012; EFSA, 2013 (revised 2014)).

Studies on entire honey bee colonies are of special importance for pesticide testing. They reflect the most realistic scenario of exposure of the different life stages, and integrate the social stress buffering mechanisms of the species. They therefore are the ultimate way to judge whether effects observed in individual larvae or adults are economically and ecologically relevant (reviewed for the

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case of neonicotinoids in Fairbrother et al., 2014). Classical endpoints of colony-level studies are direct elements of colony vitality, and usually include the amounts of bees and brood, brood survival, forager mortality, and overwintering success ("primary endpoints", EFSA, 2013 (revised 2014)).

Studies at the colony level face special inherent problems – they are expensive, time-consuming, often lack precision due to difficulties with the standardisation of subjects (colonies), and are generally of low repeatability because of environmental factors that can only partly be controlled (EASAC, 2015; Fairbrother et al., 2014). Some of these problems could be alleviated if additional/alternative colony-level endpoints (biomarkers) could be found that are faster, easier, and/or more precisely/reliably measured than the primary endpoints. While short-term effects on colony strength can be measured relatively soon after the end of exposure, assessment of overwintering success is inherently time consuming. Effects on overwintering success are also particularly sensitive to interactions with uncontrollable environmental factors, because the timespan during which these factors can act is very long (usually >6 months). Secondary endpoints/biomarkers that are predictive of colony overwintering strength (either by themselves, or in combination with classical indicators measured shortly after exposure) would therefore be of particular interest. Although they would likely be subject to interactions with environmental effects as well, the nature of these interactions may differ for different indicators, so that they can be expected to add information to models predicting overwintering strength. The problem with secondary endpoints however is that it is hard to judge of their economic and ecological meaning, i.e. their relationship with primary endpoints/protection goals (EPA, 2012).

In the present study, we screened potential secondary endpoints for the measurement of insecticide-induced stress in bee colonies. Our approach was to induce varying levels of insecticide stress, and measure the reactions of the prospected endpoints, in order to identify those that show colony-level sensitivity for the types of insecticides tested and could therefore be useful for mechanistic studies of insecticide effects at the colony level. Moreover, the relevance of observed effects for colony productivity and vitality was assessed by studying the relationship between the tested secondary endpoints and the primary endpoint overwintering strength. Because our approach required that widely varying levels of stress were applied to the experimental colonies, insecticides were administered at concentrations that were partly higher than those to which bees would be exposed in non-experimental field settings. The two substances used, the neonicotinoid imidacloprid and the carbamate compound fenoxycarb, exhibit two very different modes of action (neurotoxic effect vs. hormonal dysregulation). Their choice was additionally motivated by the fact that imidacloprid, as a neonicotinoid, is part of the group of insecticides that have been at the centre of the controversy regarding the adequacy of current testing procedures (recently reviewed by Blacquière et al., 2012; Fairbrother et al., 2014), while fenoxycarb is frequently used as positive control in toxicological studies in *Apis mellifera* and is known for long-lasting effects on colony development (EASAC, 2015; OECD, 2014; Thompson et al., 2005). The biochemical and morphometrical indicators tested were chosen to reflect foraging efficiency, brood rearing, immune status and age composition of the colony. Many of them were inspired by research on sublethal pesticide effects in *Apis mellifera*. These include the indicators of immune status, development of the hypopharyngeal glands, and learning performance, all three known to be affected by both neonicotinoids (Aliouane et al., 2009; Di Prisco et al., 2013; Hatjina et al., 2013) and insect growth regulators (Abramson et al., 2004; Heylen et al., 2011; Pinto et al., 2000).

2. Materials and methods

2.1. Colonies

Colonies used were artificial swarms set up with 2 kg of bees of mixed age, previously treated against *Varroa destructor* with oxalic acid (40 mL of a 3.5% w/v solution in 50% w/v sucrose), and young, naturally-mated queens of the subspecies *A. m. carnica*. They were installed in hive boxes containing 11 frames of 825 cm^2 (German Standard). In order to increase the representativeness of results, daughters of nine different mother queens were used. Sister groups were of different size 5–11 and had been mated in different locations. These queens were randomly attributed to treatment groups. The colonies were allowed to develop for three weeks before the start of insecticide exposure, to make sure that all brood stages were present at the start of the experiment.

2.2. Insecticide exposure and blinding of study

The aim of the exposure was not to evaluate effects of the insecticides under field-realistic conditions, but to cause measurable stress at variable levels in order to compare the sensitivity of stress indicators, as well as their relationship to overwintering success. The methodology followed for insecticide exposure was modelled on protocols used for semi-field tests for honeybee risk assessment (EPPO, 2010), modified to allow greater control of the dose and concentration administered. In order to allow direct exposure of forager bees, but still make sure that each colony only received the intended treatment, each hive was placed within a tent of 4×5 m, containing nearly no flowering plants. Before placing the hives inside the tents, stores of honey and pollen were checked to make sure that they were still similar, and that no starvation could take place. The insecticides were offered in dissolved form in 50% w/v sucrose. Imidacloprid (98.7% pure; HPC, Cunnersdorf, Germany) was directly dissolved in the sucrose solution, while fenoxycarb (99.3% pure; HPC, Cunnersdorf, Germany) was added from stock solutions in dimethyl sulfoxide (DMSO; final concentration of solvent in the sucrose solution 0.5% v/v). In order to maximise uptake and at the same time allow direct exposure of forager bees, the total feeding volume of 1 L/day was split in two portions fed in separate feeders. Of these, one was placed within the hive box, the other outside of it. The solution in the feeders was renewed daily and the volume left over from the previous day was measured for the calculation of insecticide uptake. Feeding lasted for five consecutive days. Three different concentrations of imidacloprid (1000, 200 and 50 μg/L) and three of fenoxycarb (80, 20 and 5 mg/L) were administered. A further group was fed with pure sucrose solution, containing neither insecticides nor DMSO (control). For comparison, field-relevant concentrations of imidacloprid in nectar are in the area of 0.7 to 10 μg/L (Cresswell, 2011). Nectar concentrations of fenoxycarb can be expected to be low because of its low water solubility, but concentrations in pollen of plants treated at field-realistic doses during blossoming are in the range of 7.5 to 217 mg/kg (Gretenkord and Drescher, 1996, as cited in Tasei, 2002). Each concentration of each of the two insecticides was fed to a group of 9 colonies, which later was spread evenly over the three apiaries. Together with the 9 control-colonies-63 colonies were used in the study. Because of logistical limitations, these 63 colonies had to be established, exposed and observed in two batches of 31 and 32 colonies, with an offset of one week. Since it was not possible to divide the nine colonies of each treatment and the control evenly into two groups, each treatment and the control were represented by either four or five colonies in each of the two batches. After the end of insecticide feeding, the tags on the hive boxes were exchanged by a person otherwise not involved in the experiment (and not employed by any of the participating

institutions), so that all measurements of putative stress indicators were performed without knowledge of treatment group affiliation of colonies. The information on treatment group affiliation was only released to the authors of the study after completion of measurements on the colonies. The size of flight entrances was reduced to a minimum in order to reduce the risk of robbing between colonies.

2.3. Apiaries

After insecticide exposure, colonies of all six treatment groups and the control group were evenly split among three apiaries by the same person who had re-named the colonies. The three apiaries were all situated in Eastern Germany, in semi-natural landscapes bordering agricultural areas, offering sufficient and varied pollen and nectar for colony development, and were >5 km away from the site where exposure had taken place. They were handled according to normal beekeeping practice, except that no honey, combs or bees were removed or exchanged. Treatments against *Varroa destructor* were performed in late summer (August 22 and August 26; 60% formic acid at a dose of 2 mL/comb covered with bees) and during the winter (December 20; 3.5% oxalic acid; 10–25 mL/colony, depending on the size of the winter cluster). The numbers of dead mites falling onto bottom boards after the winter treatment was counted weekly for three weeks, and the sum of these counts was later used to verify whether varroosis had influenced overwintering strength.

2.4. Collection of samples for the measurement of primary and secondary endpoints

The number of bees and of open and capped brood cells, as well as the amounts of honey and pollen present in the hives, were estimated by the Liebefeld-method (Delaplane et al., 2013; Imdorf et al., 1987). These estimations took place one, eight and 38 weeks after the end of insecticide administration (summer, autumn, and post-overwintering estimations). For the measurement of candidate indicators of insecticide-induced stress, samples of hive bees, foragers, brood and hemolymph were taken. The methodology and exact timing of sampling are described in Table 1. With the exception of bees from the winter cluster, collection of samples was roughly synchronized with the summer and autumn estimations of primary endpoints, and the collected materials will therefore be referred to as summer- or autumn-samples hereafter. Deviations from this rule resulted either from logistical limitations (the slightly precocious timing of the second sampling of PER-bees, Table 1) or were motivated by the nature of measurements to be performed on the samples (for example, it would not have been reasonable to expect numbers of adult worker ovarioles to be modified by the insecticides one week after feeding, but effects on directly exposed foragers seemed most likely to manifests themselves relatively shortly after exposure).

2.5. Measurement of primary and secondary endpoints

In total, 33 measurements were performed on the experimental colonies, including primary endpoints (number of bees, amounts of brood, honey and pollen) as well as candidate secondary endpoints. Choice of the latter aimed at including all vital functions of the honeybee colony. The set of endpoints selected included indicators known or assumed to be linked to the age and health status of foragers (size of hypopharyngeal gland-acini, metabolic and immune enzymes), to the quality of brood rearing (chemical composition of brood and jelly, fluctuating asymmetry in adults), and to the health and immune status of hive bees (hemolymph vitellogenin, immune enzymes, ovary development and survival in a cage test). Table 2 summarizes the measurements performed, and Supplementary Table 1 gives information on their physiological significance as well as reasons for their inclusion in the study. Most of the protocols followed are contained in Wegener et al. (2016), with the exception of the following:

1. **Wing asymmetry** was determined as described by Schneider et al. (2003) and vanEngelsdorp et al. (2009), except that a different software was used for the determination of the coordinates of nervure from scans of the forewings (ImageJ; downloaded from imagej.nih.gov/ij/). For each colony and sampling date, averages of the values obtained from 15 forager bees were calculated.
2. **The number of hive bee ovarioles** was determined by dissecting ovaries under saline (1.55% NaCl in water) and counting ovarioles at 25 x magnification. Average numbers from 15 surviving bees of the hive bee caging experiment (Wegener et al., 2016) were used.

Table 1
Dates and procedures of sample collection.

Sample Nb.	Designation	Description/sampling method	Days after end of insecticide-administration	
			Summer samples	Autumn samples
I	Brood	Piece of brood comb (approx. 30 cm^2), containing larvae of stages 2–3	21	64
II	Shock-frozen hive bees	2×15 bees from brood combs. Taken at random but newly-hatched workers as well as individuals with worn wings or bald abdomen avoided. Shock-frozen by spraying with liquid propane/butane (SolidoFix, Roth, Germany).	21	64
III	Living hive bees	A further group of 30 workers, placed in a cage (Wegener et al., 2006) for tests of worker fertility/longevity	21	64
IV	Hemo-lymph	Pools of hemolymph collected with glass capillaries from 10 bees (1 μL/bee), taken from a brood comb. Directly expulsed into ice-cold Tris-buffer (Wegener et al., 2009) and frozen on dry ice.	21	64
V	Forager bees	2×10 forager bees, collected at the hive entrance and identified by the presence of pollen loads. Shock-frozen like the hive bees.	8	64
VI	PER-bees[a]	20 bees for learning experiment, from the outermost comb of the colony (where usually mostly foragers can be found). Sampled on five consecutive days (four per colony and day). Because of logistic limitations, these samples were collected only from one of the three apiaries.	22–28	41–47
VII	Winter bees	20 bees from the winter cluster, frozen on dry ice		194–201

[a] PER: proboscis extension reflex.

Table 2
Endpoints measured in the experimental colonies.

Abbreviation	Indicator	Measured from sample[a]	Time of sampling/ measurement[a]	Methodology
nb bees	Number of bees in colony	colony	1, 2	Liebefeld method[b]
nb capped brood	Number of capped brood cells	colony	1, 2	Liebefeld method[b]
pollen	Number of pollen cells	colony	1, 2	Liebefeld method[b]
nb brood_nb bees	ratio between number of capped brood cells and number of bees	colony	1, 2	Liebefeld method[b]
honey	Number of honey cells	colony	1, 2	Liebefeld method[b]
br_%H2O	Relative composition of larvae (stage 2–3) and surrounding jelly	I	1, 2	Gravimetric
br_%lipid				
br_aminoac	Concentration of essential amino acids in larvae and surrounding jelly	I	1	HPLC
hb_HDA	Concentration of 10-hydroxy-10-decenoic acid	II	1, 2	HPLC
hb_vitellogenin	Concentration of vitellogenin in bee hemolymph	IV	1, 2	ELISA
hb_PFK	Activity of phosphofructokinase	II	1, 2	photometric
hb_GST	Activity of glutathion s-transferase	II	1, 2	photometric
hb_POX active	Activity of phenoloxidase	II	1, 2	photometric
hb_GOX	Activity of glucose oxidase	II	1, 2	photometric
hb_head prot1	Peaks of extracted head proteins	II	1, 2	HPLC, from head extracts of bees
hb_head prot2				
hb_head prot3				
hb_head prot4				
hb_head prot5				
hb_ovary asym	Asymmetry of left and right branches of ovary	III	1, 2	Counting of ovarioles
hb_ovary dev	Ovary activation under queenless conditions	III	1, 2	Micrographic measurement of largest oocyte after 2 weeks of queenlessness
hb_ovarioles	Number of ovarioles (left+right)	III	1, 2	Counted under microscope
hb_wingasymm	Asymmetry of wing nervature	III	1, 2	Micrographic measurement
hb_cage survival	Survival of caged hive bees	III	1, 2	2 weeks caging experiment
fb_acini	Diameter of forager bee hypopharyngeal gland acini	V	1, 2	Micrographic measurement
fb_learning	Learning capacity of presumed forager bees	VI	1, 2*	Proboscis extension reflex-assay
fb_memory	Long-term memory of presumed forager bees	VI	1, 2*	Proboscis extension reflex-assay
fb_PFK	Phsphofructokinase-activity in thoraces	VI	1, 2	photometric
fb_ACO	Aconitase-activity in thoraces	VI	1, 2	photometric
fb_POX	Phenoloxidase-activity in thoraces	VI	1, 2	photometric
fb_GOX	Activity of glucose oxidase in head extracts	VI	1, 2	photometric
wb_%H2O	Body composition of winter bees	VII	3	Gravimetric
wb_%lipid				

* Measured in one out of the three apiaries only.
[a] Description of sampling dates+techniques in Table 1. 1: summer samples; 2: autumn samples.
[b] The Liebefeld-method is a semi-subjective procedure for the determination of colony strength, based on inspections of each comb (Imdorf et al., 1987).

2.6. Analysis of residues

Samples of honey and comb were taken at the time of the autumn estimations in order to see whether bees and brood sampled at this time were still directly exposed to the insecticides, and whether an unintentional spread of the insecticides between the treatment groups had occurred. Fenoxycarb-residues in larvae were measured in three colonies from each of the treatment groups that received fenoxycarb, as well as from the control. Fenoxycarb in wax was measured in samples from 3 colonies from all six treatment groups (both the three fenoxycarb-groups and the three imidacloprid-groups) and the control. Concentrations of fenoxycarb and imidacloprid were determined in honey samples from three colonies per imidacloprid-receiving treatment group and the control. The analyses were performed at the Julius-Kühn-Institute, Berlin. The target substances were extracted from honey and wax with an acetone/water-mixture (3:1, v/v). After homogenisation followed by centrifugation an aliquot of the extract was removed and after addition of sodium chloride-solution transferred onto a ChemElut® cartridge for solid-liquid-extraction. The samples were eluted with dichloromethane and the eluates evaporated to dryness. The residual extract was taken up with acetonitrile containing the internal standards, dissolved again and then put into the freezer (−18 °C) overnight. On the next day, the samples were filtered cold (syringe filter: PTFE 0.2 μm).

Identification and quantification of the residues in the samples were carried out with LC-MS/MS. The system used was a Prominence UFLC XR HPLC (SHIMADZU) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (4000 Q TRAP®; AB SCIEX) equipped with an electrospray ionisation (ESI) source. Fenoxycarb and imidacloprid were measured in the positive mode; additionally, imidacloprid-5-hydroxyl and imidacloprid-olefin were measured in the negative mode. The substances were identified by their retention time and three multiple reaction monitoring (MRM) transitions. The residues in the samples were quantified using reference standards in matrix (concentrations: 0.1, 0.5, 1, 5, 10, 25, 50 and 100 pg μL^{-1}) and deuterated internal standards. The results are averages of duplicate injections of sample extracts.

The method was validated on the basis of the recoveries obtained for control samples fortified with the target substances at the fortification levels of 1.0, 5.0 and 10 $\mu g\ kg^{-1}$. The recoveries for imidacloprid in honey were between 102% and 107% (4–10% relative standard deviation (RSD)) and the limit of quantification (LOQ) was set to 1.0 $\mu g\ kg^{-1}$. The recoveries for fenoxycarb in wax were between 86% and 98% (5–12% RSD) and for honey 111–124% (2–7% RSD). For both sample materials the LOQ was set to 1.0 $\mu g\ kg^{-1}$.

2.7. Data analysis

The data analysis aimed at answering three questions:

a. Which endpoints are sensitive to the insecticides applied?
To clarify this question, ANOVA was performed separately for each of the two insecticides+control, using the software package SPSS (IBM, 2011). The model included the factors insecticide concentration and apiary, as well as their interaction. Nonparametric ANOVA (Wilcoxon-test) was used in cases where homoscedasticity and/or normal distribution of residues could not be achieved even after data transformation.
b. Are insecticide-sensitive endpoints relevant for overwintering strength?
To test this, regressions were calculated, using all indicators found to be influenced by the insecticides in step a. as predictors, and the number of bees after overwintering as the dependent variable. Separate models were calculated for each of the two insecticides+control. Variables for which significant insecticide-apiary interactions had been detected were also included as predictors. In the case of imidacloprid, where the dependent variable (number of bees after overwintering) was normally distributed, linear regression was used. For fenoxycarb, which induced mortality in a number of colonies (overwintering strength=0), the predicted variable was transformed to an ordinal scale, and ordinal regression (applying SPSS-PLUM; IBM, 2011) was used. In order to keep track of the apiary-effect, the variable nominal variable "apiary" with three categories was transformed into two binary dummy variables (apiary1, false or true, and apiary2, false or true), in order to allow its inclusion as predictor (IBM, 2011).
c. Are variations in any of the secondary endpoints relevant predictors of colony development, regardless of whether they reflect effects of insecticides, apiary, or uncontrolled factors?

To determine which of the endpoints had the greatest predictive power for overwintering strength, regardless of whether these indicators were influenced by fenoxycarb/imidacloprid or not, data from all colonies in the experiment were pooled. All endpoints (not only the ones influenced by the insecticides) were ranked with regard to their predictive power for the number of bees after overwintering. For this, we used the random forest-method, implemented in the R-software-package of the same name (Breiman et al., 2014). After inspection of the rankings, the five highest-scoring endpoints were used as predictors of the number of bees after overwintering (transformed to an ordinal scale) in ordinal regression. In order to avoid redundancy of predictors, Pearson-correlations between them were checked. In cases where the correlations between two endpoints were >0.5, the endpoint ranking lower in the random forest-analysis was eliminated from the model.

All analyses under points a. to c. were performed separately for the summer and autumn samplings, i.e. the two dates at which most of the secondary end points were measured in the colonies (Table 1).

2.8. Partial replication of the experiment

In order to test the repeatability of effects observed in the above-described experiment, a partial replication was performed the following year, using three groups of 10 colonies each (imidacloprid 50 μg/L, fenoxycarb 20 mg/L, control). On July 12, these 30 colonies were all installed in one apiary. Shortly afterwards, a long period of severe nectar shortage set in which led to robbery (removal of food stores by foreign forager bees) from weak colonies. By the end of August, 4 out of the 10 fenoxycarb-treated colonies had collapsed and several others were found to be deprived of almost all of their honey stores. As colonies of the remaining groups must be expected to have taken up the majority of the administered insecticide from the robbed hives, and as the effects of robbery on both the victims and beneficiaries likely outweigh those of all other stress factors, results from this experiment have to be regarded as invalid.

3. Results

3.1. Insecticide uptake

The concentration of fenoxycarb in sucrose solution did not significantly influence the volume of solution taken up during the five days of feeding (ANOVA; df=36; F=2.0; P=0.12; Fig. 1). Total doses of fenoxycarb taken up per colony averaged 192.5 ± 57.6, 58.7 ± 13.7, and 15.6 ± 2.7 mg (means ± SD) for the concentrations

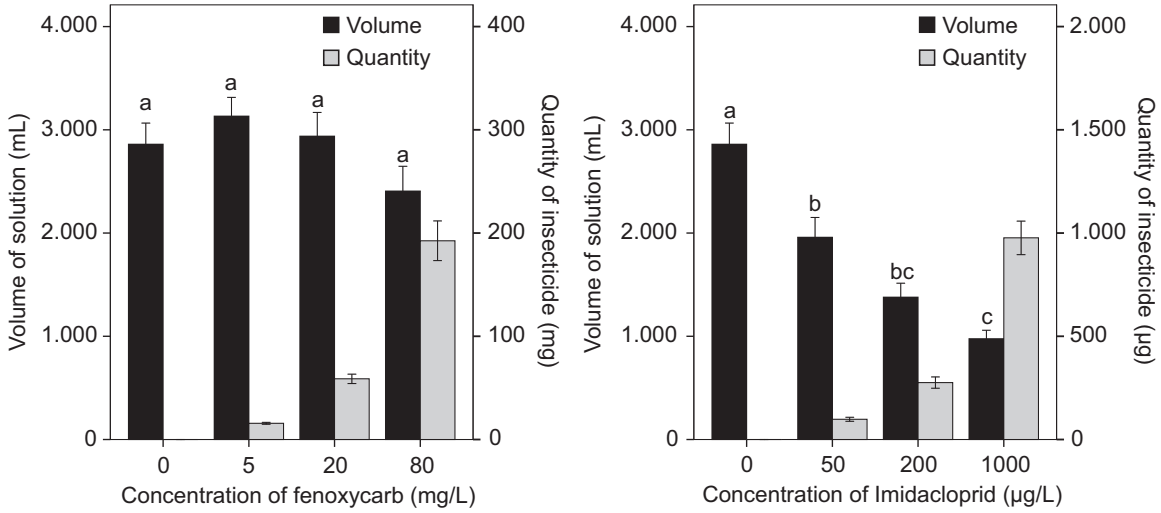

Fig. 1. Uptake of insecticide-containing sucrose solution by experimental colonies. Colonies were placed inside tents for administration of the sucrose solution. The solution was offered in two feeders, one inside the colony and one outside of it, and the volume taken up was summed. Each bar represents the mean ± s.e. of nine colonies. Data of (fenoxycarb-treatments and control) and of (imidacloprid-treatments and control) were analysed separately by ANOVA with Tukey-post hoc-tests. Different letters above bars represent significant differences ($P<0.05$).

of 80, 20, and 5 mg/L. By contrast, presence of imidacloprid strongly reduced the volume of solution taken up in a seemingly dose-dependent way (df=36; F=25.6; $P<0.001$). Nevertheless, the total dose consumed per colony was still higher in the treatments receiving higher concentrations (Fig. 1; 976.7 ± 244.4, 275.8 ± 81.5, and 97.9 ± 29.6 μg for the concentrations of 1000, 200 and 50 μg/L).

3.2. Presence of insecticides in exposed and control colonies at the time of the autumn samplings

Fenoxycarb was found in wax from fenoxycarb-treated colonies at concentrations between 54.5 and 5808.0 μg/kg. Traces of it were also found in wax from several colonies from other treatment groups, but only up to a concentration of 48.1 μg (median 2.6 μg/kg). Larvae of treated colonies contained between 5.2 and 509.5 μg/kg of the substance, compared to 0 in the control group. Concentrations in honey, as far as they were detectable, were below 1.5 μg/kg in all samples. Imidacloprid was only found in honey from imidacloprid-treated colonies (0–21.5 μg/kg; median=1.1 μg/kg) and not in the control. The metabolites of imidacloprid were not found in the honey samples.

3.3. Effects of the insecticides on primary indicators

All control colonies survived the winter. One colony was lost from each of the three groups receiving imidacloprid. For the fenoxycarb-treated groups, numbers of lost colonies were 5 (80 mg/L), 2 (20 mg/L), and 0 (5 mg/L). Three colonies lost their queen during the summer, two from treatment groups receiving imidacloprid (1.000 and 200 μG/L) and one from the group receiving 5 mg/L fenoxycarb. They were allowed to re-queen.

The effects of fenoxycarb and imidacloprid on primary endpoints at the time of the two sampling periods (8–28 and 41–64 days after the end of insecticide administration) are summarised at the top of Table 3. Results of one colony were withdrawn from the dataset, because its overwintering strength was >2 standard deviations greater than the mean of its treatment group (group mean: 2776, S.D.2728; value of outlier=9338).

Both insecticides clearly influenced the development of the colonies, with fenoxycarb leading to a strong reduction of the amount of capped brood at the time of the summer estimations ($P<0.001$), and of the number of adult bees in autumn ($P<0.001$). Imidacloprid led to a reduction of the number of adult bees in autumn ($P<0.01$). Honey stores inside colonies were also reduced by both imidacloprid (summer estimations; $P<0.01$) and fenoxycarb (autumn estimations; $P<0.05$). Imidacloprid feeding also affected overwintering strength (=number of bees found in April; ANOVA with factors insecticide concentration and apiary; n=35, F=4.64, $P=0.01$; Fig. 2). Surprisingly, high concentrations of imidacloprid appeared to affect overwintering strength less strongly than low concentrations. There were also significant effects of the apiary (F=16.76, $P<0.001$) as well as interactions between imidacloprid treatment and apiary (F=3.18, $P=0.02$). In the case of

Table 3
Effects of fenoxycarb and imidacloprid on primary and secondary endpoints.

	Fenoxycarb						Imidacloprid					
	Summer			Autumn			Summer			Autumn		
	Treatment	Apiary	Interaction	Treatment	Apiary	Interaction	Treatment	Apiary	Interaction	Treatment	Apiary	Interaction
nbbees	n.s.	n.s.	n.s.	↘	n.s.	n.s.	n.s.			↘	±	±
nbbrood_nbbees	↘	±	n.s.	↗	n.s.	n.s.	n.s.			n.s.	±	n.s.
nbcappedbrood	↘	±	n.s.	n.s.			n.s.	±	n.s.	n.s.	±	n.s.
pollen	n.s.			n.s.	±	n.s.	n.s.	±	n.s.	n.s.	n.s.	n.s.
honey	n.s.	±	n.s.	↘	±	n.s.	↘	±	n.s.	n.s.	n.s.	n.s.
hb_wingasymm	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
hb_vitellogenin	n.s.	±	n.s.	↘	n.s.	n.s.	n.s.	±	n.s.	n.s.	n.s.	n.s.
hb_ovarydevel	n.s.	n.s.	n.s.	n.s.			n.s.	n.s.	n.s.	n.s.	±	n.s.
hb_ovaryasymm	n.s.	n.s.	n.s.	n.s.			n.s.	n.s.	n.s.	n.s.		
hb_ovarioles	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	↗	n.s.	n.s.
fb_learning [2]	↗	[2]	[2]	n.s.	[2]	[2]	n.s.	[2]	[2]	n.s.	[2]	[2]
fb_memory[2]	↘	[2]	[2]	n.s.	[2]	[2]	n.s.	[2]	[2]	n.s.	[2]	[2]
fb_acini	n.s.	±	n.s.	n.s.			n.s.	±	n.s.	↗	±	n.s.
fb_PFK	n.s.			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
fb_ACO	n.s.	n.s.	±	n.s.			n.s.			n.s.		
fb_POX	n.s.			n.s.	n.s.	n.s.	↘	n.s.	n.s.	n.s.	n.s.	n.s.
fb_GOX	n.s.	n.s.	n.s.	n.s.			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
hb_POX	↘	±	n.s.	n.s.			n.s.	±	n.s.	n.s.	n.s.	n.s.
hb_GOX	n.s.	±	n.s.	n.s.	n.s.	n.s.	n.s.			n.s.	n.s.	n.s.
hb_GST	n.s.			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
hb_cagesurvival	n.s.			n.s.			n.s.			n.s.	n.s.	n.s.
hb_headprot1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	±	n.s.	n.s.	n.s.	n.s.
hb_headprot2	n.s.	n.s.	n.s.	n.s.			n.s.	n.s.		n.s.		
hb_headprot3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.		n.s.		
hb_headprot4	n.s.			n.s.			n.s.	n.s.		n.s.	n.s.	n.s.
hb_headprot5	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
hb_HDA	n.s.	n.s.	±	n.s.			n.s.	n.s.		n.s.	±	n.s.
br_percwater	n.s.			n.s.			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
br_perclipid	n.s.	n.s.	n.s.	n.s.			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
br_aminoac	n.s.	n.s.	n.s.	n.a.			n.s.	n.s.	n.s.	n.a.		

n.a.: not analysed; n.s.: not significant.
↘↘↘: significant negative treatment effect ($P<0.05$, 0.01, or 0.001).
↗↗: significant positive treatment effect ($P<0.05$ or 0.01).
± ± ±: significant treatment-apiary interaction or effect of apiary ($P<0.05$, 0.01, 0.001).
[2]Only measured in one of the three apiaries.
Empty fields in the columns "apiary" and "interaction" signify that data could not be treated by parametric methods (ANOVA), so that no two-way analysis was possible.

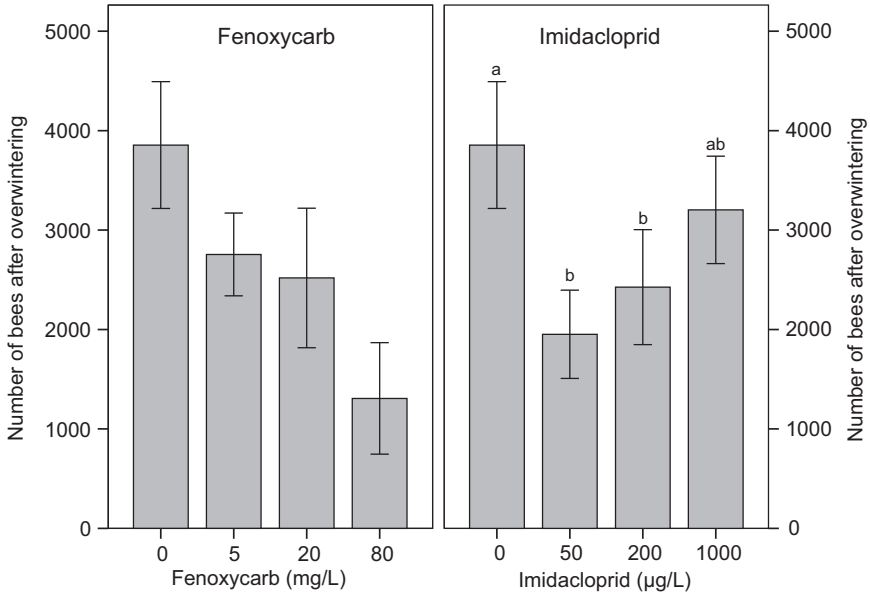

Fig. 2. Effect of insecticide-feeding in early summer on colony strength after overwintering. Each bar represents the mean ± s.e. of eight to nine colonies, placed at three different apiaries. Data for imidacloprid were analysed by two-factorial ANOVA with post-hoc Tukey-tests for the factor treatment, and different letters above bars represent significant differences ($P < 0.05$). A similar treatment of the fenoxycarb-data shown in the graph was not possible because the collapse of colonies led to non-normal distributions. Instead, an analysis of fenoxycarb-effects on colony growth (number of bees after overwintering minus number of bees before insecticide administration) was performed (see section "Effects of the insecticides on primary indicators" for details).

fenoxycarb, numbers of bees after overwintering could not be analysed by parametrical methods because the collapse of treated colonies led to non-normal distributions (seven fenoxycarb-treated colonies with 0 bees), so that colony growth (=difference between the numbers of bees after overwintering and before insecticide administration) was used instead. Fenoxycarb-administration led to significant reductions of colony growth (ANOVA, n=36, F=5.78, P=0.004), which was also affected by the factor "apiary" (F=5.43; P=0.01).

A separate ANOVA also including the number of dead varroa mites after the winter treatments (entered as mites collected over three weeks/1000 bees) as a covariate confirmed that there had not been a significant influence of varroa on overwintering strength (F=0.32; P=0.58).

3.4. Effect of insecticides on secondary endpoints

These effects are summarized in the lower part of Table 3. Out of the 26 variables measured at the time of the first (=summer) sampling, only three were significantly influenced by fenoxycarb (fb_memory: $P < 0.01$; fb_learning: $P < 0.01$; hb_POX: $P < 0.05$), and only one (hb_vitellogenin; $P < 0.05$) out of the 25 measured at the time of the autumn samplings. Imidacloprid influenced one indicator (fb_POX, $P < 0.05$) at the first and two indicators (hb_ovarioles and fb_acini, $P < 0.05$) at the autumn sampling date. Differences between the three apiaries affected slightly more of the indicators (see Table 3 for details). The composition of winter bees (samples taken 194 – 201 days after administration) was not influenced by the insecticides ($P > 0.2$ in each case).

Table 4
Summary of regression models to verify the importance of insecticide-sensitive endpoints for overwintering success.

Sampling period	Insecticide	Model	Predictor	Median	Coefficient/ estimator	Standard error	P (estimator/coeff.)	P (model)
Summer	Imida-cloprid	Linear	apiary1		235.38	63.42	0.00	< 0.001
			apiary2		−11.71	98.65	0.91	
			honey	4937	0.10	0.09	0.31	
			fb_POX	89.5	−4.78	7.84	0.55	
Autumn	Fenoxy-carb	Ordinal	apiary1		2.56	1.06	0.015	0.010
			apiary2		1.84	1.04	0.076	
			nb capped brood	808	0.00	0.00	0.048	
			fb_ACO	15.5	0.07	0.10	0.49	
			hb_POX	127	0.00	0.01	0.79	
			hb_HDA	21	−0.11	0.05	0.03	
Summer	Imida-cloprid	Linear	apiary1		165.37	67.95	0.02	< 0.001
			apiary2		64.94	57.71	0.27	
			nb bees	3960	0.55	0.19	0.010	
			hb_ovarioles	6.63	196.45	133.89	0.16	
			fb_acini	9.51	−140.79	316.24	0.66	
Autumn	Fenoxy-carb	Ordinal	apiary1		2.99	1.41	0.03	< 0.001
			apiary2		3.39	2.03	0.09	
			nb bees	3027	0.00	0.00	< 0.001	
			honey	21,005	0.00	0.00	0.703	
			hb_vitellogenin	4.31	0.26	0.35	0.456	

Overwintering success was expressed as the number of bees present in April, and was classified for use in ordinal regression. Only those endpoints were included as predictors that had shown to be influenced by the insecticide (see Table 2 for these influences). The factor "apiary" (ordinal predictor with three categories) was included in the form of two binary dummy variables ("apiary1" and "apiary2").

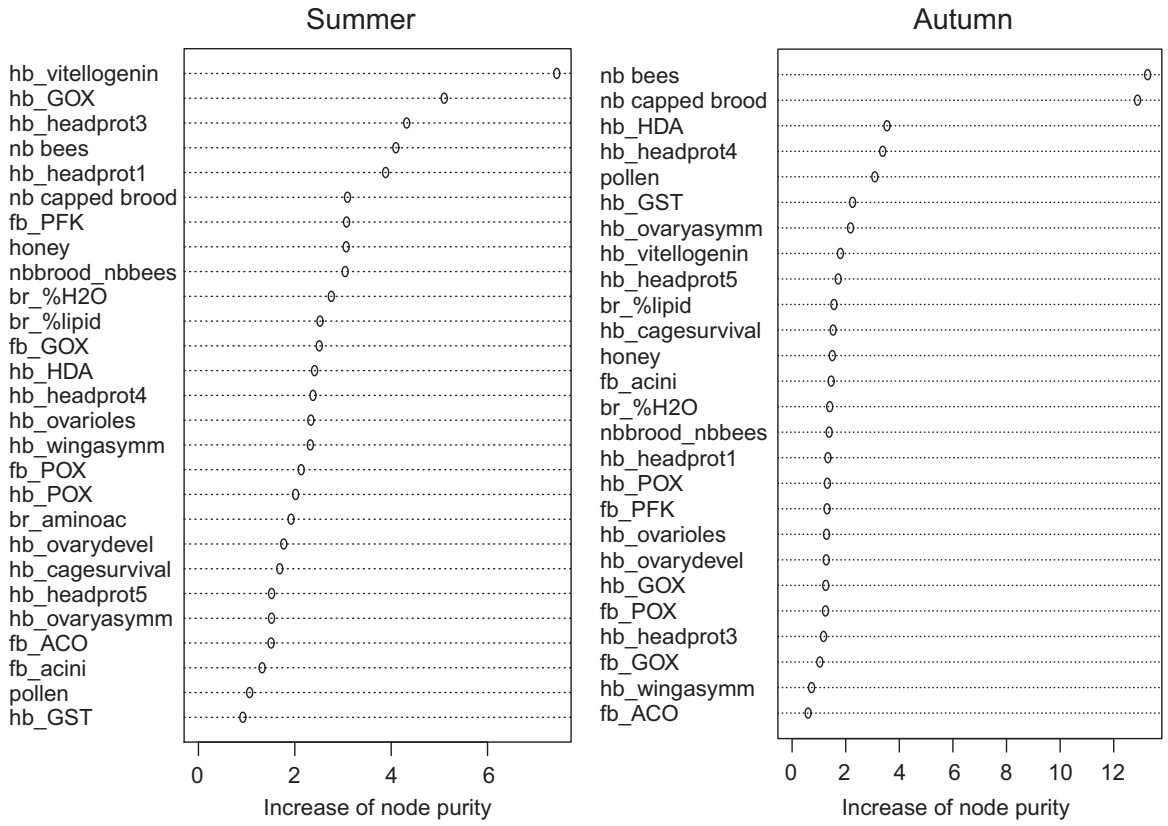

Fig. 3. Ranking of endpoints by their suitability as predictors of overwintering strength. The rankings were calculated by the random forest-method, using the number of bees after overwintering as the predicted variable. "Increase of node purity" is a measure of how strongly the accuracy of the prediction model is increased by the addition of a variable. Abbreviations of endpoints are explained in Table 2.

3.5. Relationship between endpoints and overwintering strength

Regressions aiming at predicting the number of bees after overwintering from endpoints that were influenced by the insecticides (either directly or through interactions with the apiary) are summarized in Table 4. Indicators "honey" and "fb_POX", found to be influenced by imidacloprid (summer samplings, 8–21 days after exposure), had no significant effects on the number of bees after overwintering ($P > 0.05$). In the case of fenoxycarb, coefficients of both the primary endpoint "nb capped brood" and the secondary endpoint "hb_HDA" were marginally significant ($P = 0.048$ and 0.033, respectively), while those of "fb_ACO" and "hb_POX" were not. As for the results from sampling period 2 (autumn samplings), only the effects of the two insecticides on the size of the adult bee population, which became detectable at this date, translated into an influence on overwintering strength (imidacloprid: $P = 0.01$; fenoxycarb: $P < 0.001$). Contributions of all secondary endpoints were non-significant ($P > 0.05$). There was a significant influence of the different apiaries on overwintering strength, which could be detected in all regressions.

Fig. 3 shows the ranking of *all* endpoints measured (influenced or not by the insecticides) with regard to their potential as predictors of the number of bees after overwintering, calculated by the random forest-method. For sampling period 1 (summer samplings), the top five indicators were "hb_vitellogenin", "hb_GOX", "hb_headprot3", "nb bees" and "hb_headprot1". Of these "hb_headprot1" was excluded from the regression, because it was closely correlated to "hb_headprot3" ($r = 0.87$; $n = 62$; $P < 0.001$). Of the remaining four, "nb bees", "hb_GOX" and "hb_vitellogenin" significantly contributed to the prediction of the number of bees after overwintering by the ordinal regression model shown in Table 5 ($P = 0.004$, 0.040 and 0.049, respectively), while

Table 5
Summary of ordinal regression models describing overwintering strength, using the five most informative endpoints measured at each of the two sampling periods.

Sampling period	Predictor	Median	Estimator	Standard error	*P* (estimator)	*P* (model)
Summer	nb bees	4860	0.001	0.000	0.004	< 0.001
	hb_vitellogenin	3.2	−0.360	0.183	0.049	
	hb_GOX	1.3	−0.464	0.226	0.040	
	hb_headprot3	291.2	−0.005	0.004	0.173	
Autumn	nb bees	3279	0.001	0.000	< 0.001	< 0.001
	hb_headprot4	447.3	−0.006	0.002	0.006	
	hb_HDA	21.8	−0.029	0.032	0.366	
	pollen	286.5	0.001	0.001	0.398	

The dependent variable was the number of bees after overwintering (classified). Predictors were chosen by random forest-analysis from the ensemble of all endpoints (Fig. 3). In contrast to the analyses shown in Table 3, the effects of apiary and insecticides were not included in the model and is therefore reflected in the effects of the predictors. Two endpoints were left out of the models because they were closely correlated to some of those included. This concerned hb_headprot1 (summer samplings) and nb_capped brood (autumn samplings).

"hb_headprot3" did not. At the time the autumn samples were taken, the top five predictors of the random forest-ranking were "nb bees", "nb capped brood", "hb_HDA", "hb_headprot4" and "pollen". Of these, "nb capped brood" was left out of the regression because it was strongly correlated to "nb bees" ($r=0.56$, $n=58$, $P<0.001$). Of the remaining four indicators, "nb bees" and "hb_headprot4" both contributed significantly to the ordinal regression ($P<0.001$ and $P=0.006$, respectively), while "hb_HDA" and "pollen" did not.

Sampling period 1: summer samplings; sampling period 2: autumn samplings.

4. Discussion

The special challenge with field studies on honey bee colonies lies in the fact that interactions with environmental factors can influence the results in ways that are hard to predict, and that render interpretation difficult. The failed attempt to replicate parts of our study the following year is an excellent example for this – food scarcity, together with the simultaneous presence of weak and strong colonies in the same apiary, likely caused the robbery of fenoxycarb-treated colonies and the dispersal of the insecticide they contained. The occurrence of robbery in year two prompted us to search for traces of insecticide dispersal between treatment groups in samples of honey that had been collected during the original study the year before. Although weak traces of fenoxycarb were also found in individual imidacloprid-treated colonies, the results show that in this case, dispersal of insecticide between treatments is unlikely to have influenced the results significantly.

A necessary precondition for evaluating indicators of stress is that stress really occurs. The choice of insecticide concentrations administered to colonies in the present study was therefore guided not by the aim of reproducing field-conditions, but by that of creating variable levels of stress in the treated hives. In this they succeeded – both imidacloprid- and fenoxycarb-treated colonies showed reductions in the numbers of adult bees and/or brood, and also reductions of the adult population or population growth after overwintering. Interestingly, there was an inverse relationship between imidacloprid concentration and the obtained effect on overwintering strength. Together with the observed reduction of solution uptake with increasing imidacloprid-concentration (Fig. 1), this seems to suggest that high concentrations of imidacloprid in food lead to an avoidance by worker bees. Similar findings were presented by Mayer and Lunden (1997); as cited in Decourtye and Devillers, 2010 and Dively et al. (2015) for *Apis mellifera*, and by Laycock et al. (2012) for *Bombus terrestris*. An alternative explanation may be that bees taking up syrup with high concentrations of imidacloprid are damaged in a way that partly prevents them from transporting and/or distributing the syrup.

Only relatively few of the secondary endpoints showed measurable effects of the two insecticides. This might in part be explained by the fact that the experimental design, with three different apiaries as well as Carnica queens of deliberately diverse origin, favoured robustness of results over precision of measurements. A second likely reason is that measurements on bees or brood were performed not on individually-dosed larvae/workers, but on samples collected at random from exposed colonies. While this is the only approach that is fully compatible with colony-level field studies, and also arguably the only that yields results which are representative of the colony as a whole, it is different from the methodologies used in many studies in which physiological effects even of far lower concentrations of insecticides were found (e.g Decourtye et al., 2004; Heylen et al., 2011). It is possible that some individuals from the exposed colonies were affected in physiological functions assessed by our secondary endpoints, and that this contributed to changes in primary endpoints, although *average* values of the secondary endpoints were little affected..

Those few secondary endpoints that were influenced by the treatments can a priori be seen as candidates for investigating insecticide effects at colony level. Two out of the three biomarkers affected by imidacloprid, fb_POX and fb_acini, were measured on forager bees. Together with the observation that imidacloprid reduced the amount of honey inside the colonies, this adds to findings suggesting that neonicotinoids mainly affect colony health by reducing foraging performance/increasing forager losses (reviewed in Decourtye and Devillers, 2010; see also newer data by Fischer et al., 2014; Sandrock et al., 2014). The third effect of imidacloprid was an increase of the number of ovarioles in worker bees. Such an increase can result from a deficiency of brood care (Wegener et al., 2009) and may therefore be an indirect consequence of changed within-colony demographics (Sandrock et al., 2014), possibly due to the loss of foragers.

As to the insect growth regulator (IGR) fenoxycarb, the strongest effects were observed in the secondary endpoints fb_learning and fb_memory. Fenoxycarb is an analogue of juvenile hormone (JH), which has been shown to improve learning and short-term (1 h) memory in individually-exposed young worker bees (McQuillan et al., 2014). Our study now indicates that similar effects can also be found at the colony level. Unfortunately, the relevance of these cognition-related effects for overwintering could not be assessed, because fb_learning and fb_memory were only measured in one of the three apiaries. The number of cases was therefore too low for regression analysis. Interestingly, fb_learning was correlated to the survival of caged bees from the brood nest ("hb_cage survival"; $r=-0.68$; $P=0.016$), suggesting that the improvement of learning performance by fenoxycarb was linked to a reduction in individual health/acceleration of ageing. This would be in accordance with the role of juvenile hormone as a pacemaker of ageing processes (Amdam et al., 2004).

The identification of secondary endpoints that are sensitive to the effects of insecticides may be useful for mechanistically investigating effects of substances with a similar toxicological profile on honeybee colonies. However, the existence of physiological effects alone is insufficient to evaluate the economic and ecological risk associated with the use of a given substance. For this reason, the most valuable secondary endpoints would be those that are sensitive to the class of substances in question AND of relevance for longer-term colony performance/survival, and could therefore be used to complete or even partly replace findings regarding primary endpoints (EFSA, 2013 (revised 2014)). In the present study, HDA-concentration in bee head extracts from fenoxycarb-exposed colonies was found to be both influenced by the stressor and predictive of overwintering strength. HDA is quantitatively the most important fatty acid in larval food (reviewed in Winston, 1987), and produced by the mandibular glands of nurse bees. Its concentration in hive bee heads can therefore be interpreted as an indicator for the quality of brood care. While no information exists as to direct effects of juvenile hormone analogues on HDA-production, it is possible that it is part of the set of physiological attributes of nurse bees, which together are controlled by a regulatory circuit of which JH is a central element (Amdam et al., 2004, 2005). An effect of the JH-analogue fenoxycarb on HDA-synthesis is therefore no surprise, but the fact that it was related to overwintering strength is remarkable and may indicate the usefulness of this variable for the study of IGR-effects on bee colonies.

Some of the physiological indicators (secondary endpoints) measured in hive bees could be linked to overwintering strength although they were not found to be directly influenced by the insecticides. This was the case of hb_GOX, hb_vitellogenin, and

hb_headprot4. The expression of the vitellogenin-gene was identified as a predictor of colony collapse in another study (Dainat et al., 2012). The fundamental role of vitellogenin for different aspects of honeybee physiology is well documented (Amdam et al., 2003, 2006; Guidugli et al., 2005; Nelson et al., 2007). As to hb_GOX, Glucose oxidase-expression and secretion into brood food and honey is seen as an element of social immunity (Bucekova et al., 2014; Evans and Spivak, 2010). We found in a separate study that low values of this indicator were characteristic of colonies at a terminal stage of varroosis (Wegener et al., 2016). In the present experiment however, low levels of hb_GOX were more characteristic of colonies which later overwintered strongly. A hypothesis to explain this contradiction is that GOX-expression is increased in response to stress, but breaks down when stress becomes too severe. In the varroa-study, stress levels (and average GOX-activities in worker tissues) were much higher than in the experiment described here, and only dropped shortly before colony collapse. The fact that a protein from hive bee head extracts (hb_headprot4) was also predictive of colony development seems remarkable. Although the identity of this protein is unknown, a comparison of the chromatograms of head extracts and of royal jelly suggests that most proteins from the extract may be Major Royal Jelly Proteins (data not shown).

To our knowledge, this is the biggest methodological study on secondary indicators for the evaluation of pesticide effects on bees to date. The results underline the importance of including the superorganismal level in the study of honeybee toxicology, as some of the effects that have been described from individually-exposed bees (improved learning performance after exposure to JH-analogues, reduced POX-activity in neonicotinoid-exposed bees) could be found at the colony-level as well, whereas others like the effect of imidacloprid on associative learning (Decourtye et al., 2004) appear to have been buffered by “superorganism resilience” (Straub et al., 2015). Those secondary endpoints that were found to be sensitive to either of the insecticides used here may be useful for the elucidation of the ways in which toxicants interact with bees and colonies. Moreover, we showed that HDA-production by hive bees may be an interesting early indicator of IGR-induced stress that really affects overwintering success. However, out of the 26 secondary endpoints tested here, HDA was the only to be both sensitive to one of the stressors and predictive of longer-term effects on primary endpoints. The fact that most secondary endpoints influenced by the treatments later showed to be irrelevant for overwintering strength illustrates the great resilience of the honey bee colon, although the example of the failed replication of our study also vividly shows that effects can be exacerbated by interactions with environmental factors that are hard to control or foresee. It also has to be stressed that the absence of effects on overwintering is not synonymous to the absence of ecological and economic consequences. The main conclusion of our work however has to be that secondary endpoints, at least most of those tested here, are likely of limited use for the economic and ecologic evaluation of JH-analogue or neonicotinoid effects on honeybee colonies. On the other hand, we have shown that indicators like GOX-activity and hemolymph titres of vitellogenin, measured at colony level, can tell more about colony vitality than the mere numbers of bees and brood cells. They may therefore be useful for studies involving other stressors.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2016.06.038.

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