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Article (Accepted Version)

Ratnieks, Francis L W, Balfour, Nicholas J and Carreck, Norman L (2018) [Review] Have suitable experimental designs been used to determine the effects of neonicotinoid insecticides on bee colony performance in the field? *Journal of Apicultural Research*, 57 (4). pp. 586-592. ISSN 0021-8839

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REVIEW ARTICLE

Review: Have suitable experimental designs been used to determine the effects of neonicotinoid insecticides on bee colony performance in the field?

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Summary

Laboratory studies have shown that neonicotinoid insecticides can cause sub-lethal effects on bees. Field studies are needed to determine whether these effects also occur when bees forage on flowering crops grown from neonicotinoid-treated seeds. However, for many reasons the results of field experiments may be inconsistent. For example, neonicotinoid residues in crop nectar and pollen and the availability of alternative nectar and pollen sources vary. In addition to these practical difficulties, different field experiment designs, especially different controls, address different questions. Most field studies on neonicotinoids have compared the performance of colonies adjacent to a bee-attractive crop grown from treated seeds with control colonies adjacent to the same crop grown from untreated seeds. This makes it possible to determine the cost, C , of any insecticide residues but not the overall effect of the crop, $B-C$, which includes any benefits, B , of the additional pollen and nectar. An alternative and rarely used design, only 1 of the 12 field studies reviewed, determines the overall effect of the crop by siting control colonies out of foraging range of the crop but in the same landscape. This design may be more relevant because bee-attractive crops, such as oilseed rape, are normally grown as alternatives to arable crops that do not provide bee forage, such as wheat and barley. As a result, it is possible that $B - C > 0$ (or $B - C = 0$) even if $C > 0$. The challenges and merits of alternative experimental designs are discussed in relation to practical considerations and policy making.

Key words: honey bee, bumble bee, *Apis mellifera*, *Bombus*, colony performance, neonicotinoid, oilseed rape, bee attractive crop, EU moratorium, experimental design

Short title: Neonicotinoids and design of field experiments

Introduction

Insecticides have long been known to harm bees (Hammer, 1953; Carreck 2016).

Neonicotinoids are a recent class of insecticide in use since the early 1990s (Jeschke & Nauen, 2008). In contrast to most insecticides used for plant protection, which are sprayed onto plant surfaces, neonicotinoids can be translocated within plant tissues thereby providing systemic protection (Elbert *et al.* 2008). One major application of systemic neonicotinoids is to treat seeds in order to protect young plants against insect herbivores (Elbert *et al.* 2008). A consequence of seed treatment and systemic action is that neonicotinoid residues, typically 0-2 ppb but sometimes higher (Blacqueire *et al.* 2012; Carreck & Ratnieks, 2014; Godfray *et al.*, 2014; Rundlöf *et al.*, 2015), occur in the nectar and pollen of mature plants, which are collected by foraging bees. As a result of concerns that these residues may harm bees, in 2013 the European Commission imposed a moratorium on the use of neonicotinoids as seed treatments on bee-attractive crops (Europa 2013). Among the crops affected are oilseed rape, maize, and sunflower. Following an updated assessment by the European Food Safety Authority (EFSA, 2018), it is likely that the moratorium will become a permanent ban.

The initial main evidence that neonicotinoid seed treatment of crops harms bees was indirect, from laboratory-led studies in which honey bees (*Apis mellifera*) or bumble bees (*Bombus* spp.) were fed or topically treated with insecticide. These experiments showed that the neonicotinoid levels used were not immediately fatal, but could, for example, reduce colony queen production (Whitehorn *et al.* 2012), colony population and productivity (Gill *et al.* 2012) or, in individual worker honey bees, cause navigational problems (Henry *et al.* 2012) and affect dance communication behavior and response to sugar solutions (Eiri & Nieh 2012).

However, laboratory studies may not have reflected field conditions. In particular, the doses used were usually greater than is typically recorded in the field (Carreck & Ratnieks, 2014). Furthermore, individual bees were exposed to insecticide in a single large dose rather than gradually, as would occur when a foraging bee is collecting nectar over many days (Henry *et al.* 2012; Carreck & Ratnieks, 2014). To overcome the limitations of laboratory studies, field studies that measure the performance of bee colonies foraging on neonicotinoid-treated crops are needed. A number have now been carried out, some of which indicate reduced performance of bumble bee (reduced weight gain over two months: Rundlöf *et al.* 2015) and honey bee (reduced weight gain over one year: Balfour *et al.* 2017) colonies and others which have found no performance effects (bumble bees: Balfour *et al.* 2017; Cutler & Scott-Dupree, 2014; Sterk *et al.* 2016; Thompson *et al.*, 2013; Thompson *et*

al., 2016; honey bees: Cutler & Scott-Dupree, 2007; Cutler *et al.* 2014; Hernando *et al.* 2017; Rolke *et al.* 2016).

There are several possible reasons for these contrasting findings. First, not all studies have used the same neonicotinoid insecticide, bee species or crop. Field studies have examined the effects of flowering sunflower, maize and oilseed rape grown from seeds treated with imidacloprid, clothianidin and thiamethoxam, on the honey bee (*Apis mellifera*), two species of bumble bee (*B. terrestris*, *B. impatiens*) and also one solitary bee (*Osmia bicornis*). Furthermore, the levels of neonicotinoid residues in pollen and nectar vary greatly among studies (e.g., >10 ppb [Rundlöf *et al.* 2015]; <1 ppb [Cutler & Scott-Dupree, 2014]). Residue variation has many possible causes, including mature plant size, time from planting to flowering, soil conditions and leaching, planting method, spring versus autumn planting, and the doses applied to the seeds (Balfour *et al.* 2016; Carreck & Ratnieks 2014).

These sources of variation are not simply challenges to researchers that can be overcome by appropriate experimental methods or statistical analysis. They are part and parcel of agriculture and the ecology of bee foraging on crops. In addition, the ways that crops are grown and bees forage make field research extremely challenging. Small experimental fields would be relatively easy to set up as foraging sources, but bee attractive crops are generally grown in large fields (Garbuzov *et al.* 2015, Balfour *et al.* 2017). Indeed, the fact that crops are grown in large patches is one of the reasons that flowering crops can provide significant bee forage.

An additional challenge is that bees can fly long distances to good forage sources, although the distances at which bees forage is itself highly variable and also depends on species and season (Beekman & Ratnieks 2000; Couvillon *et al.* 2014; Ratnieks & Shackleton 2015). This makes it almost impossible to ensure that all foraging is carried out on the target crop field, as may be desired in a Design 1 (see below) experiment, given that there are almost always alternative flower sources in the landscape, whether crops or wild flowers. For example, honey bees from hives located in apple orchards to assist in apple pollination visit nearby oilseed rape fields even when these are more distant (Balfour & Ratnieks, 2017). Honey bees can forage at distances of 10 km or more (Beekman & Ratnieks 2000; Ratnieks 2007) and routinely fly 1 or 2 km to rewarding flower patches (Couvillon *et al.* 2014; Garbuzov *et al.* 2015; Balfour & Ratnieks, 2017). Although bumble bee foraging distances are not as great (Ratnieks & Shackleton 2015), they are nevertheless substantial. *Bombus terrestris*, the most studied species, normally forage within 1 km of their nest (Wolf and

Moritz, 2008). Osborne *et al.* (2008) estimated maximum foraging distances of at least 1.5 km, and mass flowering crops such as oilseed rape attract bumble bees at up to approximately 1.75 km (Walther-Hellwig & Frank 2000). These foraging distances encompass areas that are many times greater than the sizes of most arable fields, For example, 9.6 km² (960 ha) for a radius of 1.75 km.

Clearly, there are practical difficulties in carrying out field studies of bee foraging on crops. However, there is another challenge that is both distinct and fundamental: the basic design of the experiment. Different designs, and especially different controls, result in experiments that address different questions. Here we draw attention to the importance of experimental design in determining the effects of insecticides on bees under agricultural field conditions, and discuss some of the resulting implications for policy and practice concerning agriculture, insecticide use and bee conservation.

Bee attractive crops and insecticides: benefits and costs to bees

Most bee-attractive crops grown from neonicotinoid-treated seeds are arable crops that are alternatives to crops such as wheat and barley that are not visited by bees. Unlike wheat or barley, a bee-attractive crop is a potential food source. Most bee-attractive crops, such as oilseed rape and sunflower, provide both pollen and nectar. Some, such as maize, provide only pollen. Flowering crops may be a significant source of forage for bees. Oilseed rape, for example, is visited by honey bees, bumble bees, and solitary bees (Rundlöf *et al.* 2015; Balfour and Ratnieks, 2017). Indeed, oilseed rape fields may increase bee abundance (Westphal *et al.* 2003) and wild bee nest productivity (Holzschuh *et al.* 2013), and beekeepers often move *A. mellifera* hives to oilseed rape for honey production (Carreck *et al.* 1997). However, when treated with insecticides, the same crop may also harm bees.

These benefits and costs are linked. A larger crop area will increase the total amount of nectar and pollen available. This will probably attract more foragers. However, it will also result in more exposure to any insecticide on the crop, whether systemic and so present in the nectar and pollen collected, or topical and so present on the surfaces contacted by foraging bees.

If an experiment is to be carried out to determine the effects of insecticide applied to bees in the field, how should it be designed? In particular, is the goal to determine the harm caused by the insecticide to the bees? That is, the cost, *C*, of the insecticide alone. Or is the goal to measure the overall effect of the insecticide-treated crop to bees, and especially to

determine whether this is positive or negative? It is possible that even if the insecticide has a cost ($C > 0$) the crop may be beneficial overall ($B - C > 0$) or that the net effect may be neutral ($B - C = 0$).

If the goal is to measure the cost, then a suitable design (Design 1, Equation 1 [Figure 1]) is to compare the performance of colonies with ready access to a field of a bee-attractive crop that has been treated with insecticide (both benefit and cost) with control colonies with access to a similar but untreated field (benefit but zero cost). The difference in performance, $P_{\text{exp}} - P_{\text{con}}$, between the experimental and control-group colonies provides an estimate of the cost under the prevailing conditions in that landscape.

$$\text{Design 1 (insecticide effect)} \quad P_{\text{exp}} - P_{\text{con}} = (B - C) - (B - 0) = -C \quad (1)$$

However, if the goal is to determine the difference between the benefit and the cost, then the control colonies should have no access to the crop (Design 2, Equation 2), but should be located nearby, just out of foraging range, so that they experience the same agricultural landscape and weather conditions. The difference in performance between the two groups of colonies is the benefit minus the cost.

$$\text{Design 2 (treated crop effect)} \quad P_{\text{exp}} - P_{\text{con}} = (B - C) - (0 - 0) = B - C \quad (2)$$

Table 1 lists 12 field studies carried out to date that collectively investigated 18 bee species x crop species combinations. Although both designs have been used, only one used Design 2 (Balfour *et al.* 2017).

No project has combined Designs 1 and 2 so that both C and $(B - C)$ can be directly estimated, and B can be indirectly estimated. In principle, two studies have done this to study the effects of foraging on oilseed rape grown from clothianidin-treated seeds or untreated seeds on honey bee (Rolke *et al.* 2016) and bumble bee, *B. terrestris*, (Sterk *et al.* 2016) colonies located within or distant from the crop. The experimental set up was suitable for investigating the insecticide effect. However, it was unsuitable for investigating the treated crop effect. This is because, at only 400m from the crop, the distant colonies were well within foraging range of the crop (Garbuzov *et al.* 2015; Balfour & Ratnieks 2017). Pollen analyses also showed that both the distant and in-crop colonies foraged heavily on oilseed

rape. To study the crop effect the distant colonies need to be far enough away to result in zero or low foraging on the crop (Balfour et al. 2017), with the bees instead foraging on alternative floral resources in the same agricultural area. It appears that in the region of northern Germany used for these studies, which were carried out using the same fields, oilseed rape is so widespread, covering 25-33% of the arable land, that it would have been difficult to locate bee colonies out of foraging range.

Discussion

What conclusions can be drawn from the results of Design 1 and Design 2 experiments?

If a Design 1 experiment shows no difference between control and insecticide-exposed colonies or individual bees, this indicates that the cost is zero and is a clear result, as it is controlled. Nevertheless, the conclusion that there is zero cost is not of fully general applicability because the data were collected under a particular set of agro-environmental conditions. It is possible that neonicotinoid residue levels in pollen and nectar were lower than may sometimes occur in that particular crop or that foraging on the target crop was reduced because abundant bee-attractive alternatives were within foraging range.

If the control colonies in a Design 1 experiment perform better, this indicates that there is a cost from insecticide exposure. However, it is premature to conclude that there is an overall cost of the treated crop. This can only be determined in a Design 2 experiment. Oilseed rape is an arable crop normally grown in rotation with other crops such as wheat and barley which do not produce nectar and seem not to be visited by bees to collect the pollen that is produced. In an agricultural landscape, a field oilseed rape grown from treated seeds may benefit the performance of a bee colony more than a field of wheat even if there is some insecticide cost. In terms of agricultural practice, a Design 1 experiment effectively compares an organically grown field of a particular crop with a conventionally grown (i.e., pesticide treated) field. However, only a small proportion, less than 1%, of oilseed rape, the major bee-attractive crop in the EU, is grown without insecticide (EuroStat 2016).

Interpreting the results of their Design 1 experiment in terms of bee conservation, Rundlöf et al. (2015) write “Hence, such insecticidal use can pose a substantial risk to wild bees in agricultural landscapes, and the contribution of pesticides to the global decline of wild bees may have been underestimated.” However, there is a gap in the logic behind this statement. The data do not show that bee colonies located beside fields of seed-treated

oilseed rape did worse than colonies located in the same landscape, with access to alternative arable crops that do not provide pollen or nectar.

Other insecticides

One consequence of the EU neonicotinoid moratorium and ban is that farmers will use alternative insecticides as these are still permitted on bee-attractive crops (Carreck & Ratnieks, 2013; Dewar 2016). Following the EU moratorium, many UK farmers experienced difficulty in protecting their oilseed rape crops from autumn pests such as the cabbage stem flea beetle, *Psylliodes chrysocephala*, and have resorted to spraying synthetic pyrethroids (Dewar 2016), leading to a 2.5 fold increase in the volume of insecticides applied in the autumn despite an 8 % reduction in crop area (Scott & Bilsborrow, 2015). Budge *et al.* (2015) report that, before the EU moratorium, oilseed rape grown from imidacloprid-treated seeds in the UK was treated with lesser amounts of alternative insecticides.

Field experiments should ideally be designed and carried out to compare alternative insecticides. In this way it will be possible to determine whether the replacement of neonicotinoids with other insecticides, or *vice versa*, benefits bees. To date, no study has investigated whether field application of neonicotinoid insecticides to bee-attractive arable crops is more or less harmful to bees than the non-systemic insecticides they have replaced. The fact that comparisons have not been made is somewhat surprising given that the use of synthetic insecticides predates by many decades the use of neonicotinoid insecticides and that most OSR grown in the EU is and has been treated with insecticides (Carreck 2017).

In making comparisons, there would also be challenges due to differences in the timing of any harmful effects on bees caused by different insecticides. Seed treatment with neonicotinoids is designed to protect the young plant against insect herbivores, while the potential harmful effects of residues in nectar and pollen can occur months later when the crop is in bloom. Bees are unlikely to be affected by alternative, non-systemic, insecticides with short residual effects that are applied to protect young plants that are not in bloom, unless there are flowering weeds in the crop field or if spray drifts onto wild flowers in the field margins. However, it is possible that harm may be caused to other non-target species that are active in the crop at that time or to the wider environment. Putting things another way, different insecticides may have harmful effects on different non-target species and at different time scales after application. Although concern about negative effects on bees was

the prime motivation for the EU moratorium, negative effects on other non-target species are also of great importance (Lopez-Antia et al, 2016).

Final conclusions

Our review shows that most field studies on the effects of bee-attractive crops grown from seeds treated with neonicotinoid insecticides follow Design 1. In isolation, these studies are insufficient to show whether a seed-treated crop is harmful to bees. This is because Design 1 experiments determine the cost but not the benefit of the crop. They cannot show, for example, that a field of oilseed rape treated with insecticide is better or worse for bees than alternative crops such as wheat and barley that do not provide bee forage.

Few Design 2 studies have been carried out. It is not clear why this is the case as the overall effect of the crop is certainly of great relevance. The predominance of Design 1 may reflect the desire to show a negative effect of neonicotinoids. As researchers who carried out the only Design 2 field trial in this review we were criticized by journal referees for carrying out an “incorrectly designed” project. This suggests that, for some reason, Design 1 is considered correct and Design 2 incorrect. In fact, neither is correct. The two designs estimate different things. What is correct, or rather what is relevant, depends on the question.

In the future, it may be advantageous to consider field studies that combine Design 1 with Design 2. To do this would require 3 treatments rather than just the 2 needed for either Design 1 or Design 2. This might be worthwhile in that the 50% additional effort would result in a deeper understanding of the effects of both the insecticide and the crop on bees.

A further logical step would be a complete 2x2 design of bee attractive crop (with and without insecticide) and insecticide (with and without bee attractive crop). To do this insecticide would also be applied to a crop that bees do not visit with bee colonies also kept at this location in addition to the 3 locations needed to combine Design 1 and Design 2. In modern intensive agriculture, in which there are usually few flowering weeds that bees might visit growing in arable crops, it is unlikely that much bee foraging will take place in a field of a crop such as wheat that is not bee attractive. Therefore, *a priori* it may be considered unlikely that there will be any effect of that crop on bees, whether cost or benefit. Indeed, many non bee-attractive crops are grown from seeds treated with neonicotinoids (Lopez-Antia et al, 2016).

In addition, it would be beneficial to determine the relative effects of additional insecticides so that neonicotinoids can be compared with alternatives. The alternatives could include insecticides replaced by neonicotinoids pre-moratorium and insecticides used post-moratorium to replace neonicotinoids.

Field studies are logistically difficult to carry out. It is easy to think of a wide range of projects and experimental designs that would provide useful information. In practice, however, resources are seldom available to do this and it will be necessary to focus on the key questions and comparisons. In terms of practicality, Woodcock et al (2016) note that onerously large levels of replication ($n = 68$ replicates) would be needed for field studies to satisfy European Food Safety Authority regulations requiring the detection of small changes, 7%, in bee colonies.

Although numerous field studies have been carried out since the EU moratorium, science-based policy recommendations on the use of neonicotinoid insecticides with respect to their possible negative effects on bees will be difficult to formulate because of the lack of field data addressing two key questions: 1. are bee-attractive crops grown from treated seeds worse for bees than alternative arable crops that are not bee-attractive; 2. are neonicotinoid insecticides worse for bees than alternative insecticides. Of course, bees are not the only non-target organisms to consider but they are the organisms whose interests have been paramount in policy making and also in the sometimes vociferous campaigning (e.g. Eisenstein, 2015).

More generally, the neonicotinoid-bee issue cannot be taken in isolation from fundamental and general questions about agriculture and human civilization. Agriculture is essential to humans yet is undeniably harmful to many wildlife species (reviewed in Robinson & Sutherland, 2002). Consider an experiment in which bee colonies are located adjacent to large wheat fields or to natural habitat and that the latter perform better. Is this then a reason for banning agriculture? Indeed, the main harm caused by mankind to terrestrial wildlife is probably the initial conversion of natural habitats into agricultural land (Mattison & Norris 2005). The debate about neonicotinoids should really be a debate about the use of agricultural pesticides in general, and how to minimize their harmful effects and maximize their benefits, within a context of an expanding human population and the need to share the planet with other species.

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Design 1. Insecticide Effect

Treatment bee colonies

Seed-treated crop

Benefit – Cost

Control bee colonies

Untreated crop

Benefit – 0 (0 Cost)

Distance sufficient
to result in low or
zero foraging by
bee colonies in the
other location

Design 2. Treated Crop Effect

Treatment bee colonies

Seed-treated crop

Benefit – Cost

Control bee colonies

No crop

0 (0 Benefit) – 0 (0 Cost)

Distance sufficient
to result in low or
zero foraging by
bee colonies in the
other location

Figure 1. The difference in the design of field experiments to quantify the effect of foraging on crops grown from seeds treated with neonicotinoid insecticides on bee colonies. Design 1 determines the effect of the insecticide. The control colonies are sited nearby, but out of foraging range, in the same landscape and next to a field of the same crop that is grown from untreated seeds, and so benefit from the nectar and pollen produced by the crop without any costs due to insecticide residues. Design 2 determines the effect of the crop. The control colonies are sited nearby in the same landscape but not next to a field of the target crop, and so neither benefit from the nectar and pollen produced by the crop nor suffer costs due to insecticide residues.

Table 1. Experimental design in field experiments investigating the effects of bee-attractive crops grown from neonicotinoid-treated seeds on the performance of bee colonies. In both Design 1 and 2, one group of colonies is in close proximity to fields grown from neonicotinoid-treated seeds. In Design 1, the control group colonies are in close proximity to fields of the same crop grown from untreated seeds. In Design 2 the control group colonies are in the same agricultural landscape but sufficiently distant to any fields of the crop to reduce foraging on that crop to a low level. *In principle, these two studies, which were carried out at the same location, combined both Design 1 and 2. In practice, however, the experimental set up did not allow the treated crop effect to be measured as the colonies more distant from the crop, 400m, were still well within foraging range and foraged heavily on the crop as shown by pollen analyses.

Author	Crop	Country	Insecticide	Bee species
Design 1				
(insecticide effect)				
Cutler & Scott-Dupree 2007	oilseed rape	Canada	imidacloprid	<i>A. mellifera</i>
Thompson et al, 2013	oilseed rape	UK	imidacloprid	<i>B. terrestris</i>
Pilling et al. 2013	oilseed rape, maize	France	thiamethoxam	<i>A. mellifera</i>
Cutler et al. 2014	oilseed rape	Canada	clothianidin	<i>A. mellifera</i>
Cutler & Scott-Dupree 2014	maize	Canada	clothianidin	<i>B. Impatiens</i>
Hernando et al. 2018	sunflower	Spain	clothianidin	<i>A. mellifera</i>
Rundlöf et al. 2015	oilseed rape	Sweden	clothianidin	<i>A. mellifera</i> , <i>B. terrestris</i> , <i>O. biocornis</i>
Rolke et al. 2016*	oilseed rape	Germany	clothianidin	<i>A. mellifera</i>
Sterk et al. 2016*	oilseed rape	Germany	clothianidin	<i>B. terrestris</i>
Thompson et al. 2016	oilseed rape	UK	thiamethoxam	<i>B. terrestris</i>
Woodcock et al. 2017	oilseed rape	Hungary, Germany, UK	clothianidin	<i>A. mellifera</i> , <i>B. terrestris</i> , <i>O. biocornis</i>
Design 2 (treated crop effect)				
Balfour et al. 2017	oilseed rape	UK	thiamethoxam	<i>A. mellifera</i>