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EFFECTS OF EXPOSURE TO WINTER OILSEED RAPE GROWN FROM THIAMETHOXAM-TREATED SEED ON THE RED MASON BEE *OSMIA BICORNIS*

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Abstract: There has been increasing interest on the effects of neonicotinoid insecticides on wild bees. In solitary bee species the direct link between each individual female and their reproductive success offers the opportunity to evaluate effects on individuals. The present study investigated effects of exposure to winter oilseed rape grown from thiamethoxam-treated seed on reproductive behaviour and output of solitary red mason bees (*Osmia bicornis*) released in 6 pairs of fields over a two-year period and confined to tunnels in a single year. When adjusted to the number of females released, there was significantly lower production of cells and cocoons per female in tunnels than in open field conditions. This difference may be due to the lack of alternative forage within the tunnels. Under open field conditions palynology of the pollen provisions within the nests demonstrated oilseed rape pollen an average of 31% at any site with *Quercus* (oak) contributing up to 86% of the pollen. There were no significant effects from exposure to oilseed rape grown from thiamethoxam-treated seed from nest establishment through cell production to emergence under tunnel or field conditions.

Keywords: Semi-field, Thiamethoxam, Osmia bicornis, Risk assessment

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INTRODUCTION

Among pesticides, by far the greatest attention in recent years has been placed on the potential adverse effects of neonicotinoid insecticides on pollinators (Klatt et al. 2016). The nitro-guanidine containing neonicotinoids, (imidacloprid, clothiandin and thiamethoxam) have been widely used as seed treatments on crops such as oilseed rape and are highly systemic, enhancing herbivorous pest control (Douglas and Tooker 2015; Kathage et al. 2017). This property may also result in their detection at low levels in pollen and nectar of flowering crops (Godfray et al. 2014; Godfray et al. 2015). The effects of exposure to these insecticides on both *Apis* and *Bombus* species have been extensively studied, particularly in the laboroatory (Balfour et al. 2017; Blacquiere et al. 2012; Cresswell 2011; Decourtye and Devillers 2010; Godfray et al. 2014; Godfray et al. 2015; Goulson 2013; Pisa et al. 2014), but there have been few studies on the effects of neonicotinoids on solitary bee species (Klatt et al. 2016; Lundin et al. 2015).

Solitary bees have been proposed as suitable test organisms for regulatory decisions on pesticide approval (EFSA 2013). However, there are no agreed guidelines for laboratory studies and very limited information on suitable semi-field or field test methods for specific species, for example foraging behaviour on model crops and “normal” reproductive output. If robust semi-field and field test methodologies can be defined, solitary bees have the potential to be a good test organism to determine potential effects of pesticides on non-*Apis* bees populations since there is a direct link between each individual female and her reproductive success (Bosch 2008; Bosch and Kemp 2004; Bosch and Vicens 2002; Bosch and Vicens 2006; Johnson 1988; Klostermeyer et al. 1973; Molumby 1997; Radmacher and Strohm 2010; Sandrock et al. 2014; Sedivy and Dormann 2014; Sedivy et al. 2011; Seidelmann et al. 2010; Strohm et al. 2000; Tepedino and Torchio 1982). Such data may then also be used to inform discussions on whether

existing regulatory risk assessment approaches based on other Hymenoptera such as *Apis mellifera* and *Aphidius* spp. are likely to be protective of non-*Apis* populations.

Field studies can be used to monitor biological responses of organisms to pesticides under more realistic exposure scenarios than laboratory studies, yet typically there are many other variables such as parasites or limited quantity or quality/diversity of forage that may affect the same parameters (Fauser-Misslin et al. 2014; Goulson et al. 2015). Only a few field-scale pollinator studies have been reported under 'real-world' scenarios as they are logistically challenging, requiring both extensive areas to prevent bees foraging on neighbouring fields containing bee attractive crops (Rundlof et al. 2015) and appropriate replication (Pilling et al. 2013; Thompson et al. 2016; Woodcock et al. 2016a). Semi-field (tunnel) studies potentially offer an approach with a higher level of control over exposure but it is currently unclear what level of replication is required and what effect confinement in itself may have on the behaviour of bees.

The present study aimed to address whether there were adverse effects on the solitary red mason bee, *Osmia bicornis,* following exposure to winter oilseed rape grown from thiamethoxam-treated seed. The study was conducted at 6 paired sites in France and Germany over the course of 2014 and 2015. Oilseed rape is reported to have a strong positive influence on the abundance of generalist solitary bees (Holzschuh et al. 2013); *Osmia* have been shown to be efficient pollinators of oilseed rape (Jauker et al. 2012) and are a recommended European regulatory test species (EFSA 2013). However, oilseed rape is not a primary source of pollen for nesting *Osmia bicornis* (Holzschuh et al. 2013; Raw 1974); instead Rosaceae and Ranunculaceae are reported as particularly favoured (Peters et al. 2016; Westrich 1989). Therefore the scale of potential exposure to the treated crop in a landscape with limited alternative crop forage was

unclear. As a low level of foraging on oilseed rape pollen was observed in the 2014 field study, tunnels were also placed on the field sites in 2015 to provide additional information under conditions where there was no alternative forage. This enabled the data from the field study with free-flying *O. bicornis* to be compared with those generated by confining *O. bicornis* in tunnels placed on the same oilseed rape fields in 2015.

MATERIALS AND METHODS

Solitary bees

Adult red mason bees *Osmia bicornis* L. (Hymenoptera; Megachilidae) were used as the test organism. *O. bicornis* is a univoltine, polylectic cavity-nesting megachilid species. Males emerge earlier than females and after mating, females forage for pollen and nectar from suitable flowering plants in order to provision a single brood cell with a pollen provision on which to deposit an egg. The egg is either fertilized, resulting in a female, or unfertilized, resulting in a male. After eclosion the larvae feeds on the pollen provision before spinning a cocoon and passing through a pre-pupal and pupal stage before eclosing as an adult (eclosion is defined as the emergence of an adult insect from a pupal case or an insect larva from an egg). The adult remains in the cocoon for overwintering before emerging in the following spring (Sandrock et al. 2014; Sedivy and Dormann 2014). Across all of the sites, incubation procedures were performed to synchronize the emergence of males and females with the flowering of the oilseed rape to ensure sufficient food resources for the development of the females and their nesting behaviour. Cocoons of *O. bicornis* from a commercial supplier (WAB – Mauerbienenzucht, Konstanz, Germany) were sourced in early March of each year and kept under cooled conditions (females and males separated) (2.6 to 2.8 ^{o}C). To initiate eclosion female and male cocoons were

incubated in a plastic box at a mean temperature of 21.1 °C for 2 to 4 days before release (Sgolastra et al. 2012).

Field sites

In 2014 the trial was conducted on winter oilseed rape at one location in France (Alsace) and two in Germany (Kraichtal and Tübingen) and in 2015 at three locations in Germany (Niefern, Tübingen (on different fields to 2014) and Celle). At each location there were two field sites (each 1.9 to 2.7 ha), one drilled with thiamethoxam-treated seed and the other with seeds treated only with fungicides (see Supplemental Data). All data were generated in compliance with Good Laboratory Practice (Cutler and Scott-Dupree 2016; OECD 1998). For the thiamethoxam-treatment the oilseed rape seed was treated at the recommended application rate of 1.5 L Cruiser OSR/100 kg seed (nominal seed loading rate: 21 µg thiamethoxam/seed, 2.5 µg metalaxyl-M/seed and 0.6 µg fludioxonil/seed; actual rates were within 10% of this (see Supplemental Data)). The untreated seed was treated with the fungicides only (metalaxyl-M and fludioxonil) at the same nominal seed loading rates (see Supplemental Data) to avoid crop failure due to disease. The sowing was conducted in the autumn of 2013 and of 2014 using calibrated commercial drilling machines at a sowing density of 3.3 kg seeds/ha for all field sites (17 g thiamethoxam /ha).

Open field study

Just before the start of flowering of the crop (growth stage BBCH 57 (Meier 2001)) eight nest units were placed in each field, sufficient to provide an excess of nest cavities for the number of female cocoons placed in the field. The front entrance of the nesting units was orientated towards the south-east in order to enhance the activity of the *O. bicornis* in the early morning and to extend the foraging activity period. The nesting units consisted of an outer

chassis which contained trays made of medium-density fibre-board, each covered by a sheet of transparent plastic, and a release tray. Each tray contained cavities (8 x 8 x 150 mm) (see Supplemental Data). The design of the trays allowed monitoring and photographing of the nest building activity over time and the removal of pollen provision for residue analysis and palynology without disturbing or damaging adjacent cells. The design of the nest units varied slightly between 2014 and 2015, based on experience of the practicality of the units in the field. In 2014 there were 15 trays each containing 20 cavities (total 300 nest holes, 2400 per field) and in 2015 there were 10 trays each containing 10 cavities (total 100 nest holes per unit, 800 per field); release rates were adjusted accordingly, see below. As *Osmia* females require muddy soil for the construction of the cells, water was added to the soil in front of the nesting units if conditions were dry.

The incubated cocoons were placed in the release boxes of the nesting units at the beginning of flowering (8 April 2014 Kraitchal; 15 April 2014 at Tübingen; 6 April 2014 at Alsace; 21 April 2015, at Niefern; 23 April 2015 at Tübingen and 29 April 2015 at Celle). This day was defined as 0 Days After Exposure (DAE). In 2014, there were two release dates with 30 females and 60 males placed in each release box at -3 DAE and an additional 120 females and 210 males at 0 DAE giving a total of 1200 females and 2160 males released per field. In 2015 this was reduced to 480 females and 720 males placed in each release tray at 0 DAE. This was based on the nest establishment experience in 2014 and the use of smaller nesting units to address problems identified with individuals locating their nests in the larger nesting units. The male:female ratio was selected to ensure sufficient males to allow the mating of all emerging females and the female density was chosen in order to have enough females for the successful colonisation of the provided nesting units which provided 2400 nest cavities in 2014 (2.0 nest

cavities per female cocoon) and 800 nest cavities per field in 2015 (1.7 nest cavities per female cocoon). At the end of flowering of winter oilseed rape (growth stage BBCH 69-71) (in 2014: 42 days after the start of exposure (DAE) at Kraitchal; 51 DAE at Tübingen; 52 DAE at Alsace; in 2015: 36 DAE at Niefern, 33 DAE at Tübingen and 36 DAE at Celle) the nest units were closed with mesh bags to protect the sealed brood cells from parasites and were left in the field sites in order to allow the undisturbed development of larvae and pupae before transporting the nest units back to the laboratory for assessment (in 2014 92 DAE at Kraitchal; 192 DAE at Tübingen; 176 DAE at Alsace; in 2015 51 - 52 DAE at Niefern, 63 - 64 DAE at Tübingen and 76 - 79 DAE at Celle). The nesting units were stored protected from the weather under ambient conditions until full maturation of the adults in the cocoons.

Semi-field study

In 2015 four tunnels (5 m wide, 12 m long and 3.5 m high and covered with a fine mesh gauze) were also set-up on each field site (control and treated) at two locations, Tübingen and Celle, however at the last location, Niefern, only one tunnel was set-up per field site due to farmer concerns about the presence of the tunnels on the farm. One nest unit was placed into each tunnel with the front entrance of the nesting units orientated towards south-east. The nesting units were the same design as used in the field and provided a total of 100 nest holes in trays with transparent covers. Water was also added to the soil in front of the nesting units if conditions were dry.

Incubated cocoons (60 female and 90 male) were placed in the release boxes of the nesting units at the beginning of flowering (the same dates as in the field). The male:female ratio was selected to ensure the mating of all emerging females and the female density was chosen in order to have enough females for the successful colonisation of the provided nesting units whilst

maintaining an excess of nest holes; the ratio of female cocoons to available nest holes was the same tunnel as in the main field (1.7 nest cavities per female cocoon). As for the main field, and on the same dates, at the end of flowering of winter oilseed rape the nest units were closed and returned to the laboratory for assessment.

Observations

Unless otherwise stated the same assessments were performed in the main field and tunnels.

Assessment of eclosion rate of released cocoons. The number of eclosed (emerged) *Osmia* males and females was assessed by counting the number of empty (eclosed) cocoons in the release trays every third day until almost all bees had eclosed (in 2014: 43 DAE at Alsace, 36 DAE at Kraitchal and 36 DAE at Tübingen; in 2015: 18 DAE at Niefern, 15 DAE at Tübingen and 21 DAE at Celle). The remaining sealed cocoons were removed at the end of these assessment periods to prevent any parasites present from emerging into the nesting units.

Nest occupation assessment. In the late evening or early morning before sunrise (i.e., during the hours of darkness when females were most likely to be present within the nest cavities) the nest occupation rate was assessed by counting the number of cavities containing adult females. In 2014 the assessments were performed between the release of the first and second batch of *Osmia* in the field (-1 DAE). Assessments were continued on 4 DAE, 7 DAE, and then every 6 days until the end of flowering. In 2015 the assessments started on 6 DAE and were conducted every third day until the end of flowering.

Cell production assessment. To evaluate the productivity (offspring production) of the female population, the cell production rate was assessed by counting produced (sealed) cells comprising both a pollen provision and an egg or larvae in the cavities on the nesting trays.

Photographs of each tray were taken through the transparent plastic cover on the tray on the assessment days in the field. The trays were labelled with a unique number that allowed the assignment to the treatment group, the replicate and the position within each of the nesting units. The photographs were evaluated by recording the number and increase in produced cells for each time period. The assessments started on 3 DAE and were continued on every third day until no further cell construction occurred at the nest sites.

Flight and foraging activity assessment. In order to evaluate whether the females were active at the nest sites, the flight intensity was assessed at the entrance of the nesting units three times per assessment day. In 2014 the females entering the cavities were counted for between one and five mins for each nesting unit, with values normalised to three mins; the range depended on the site. The assessments started between the release of the first and second batch of *Osmia* in the field (-1 DAE) and continued from 4 DAE onwards on approximately every third day until 46 DAE (Niefern) and then 50 DAE (Tübingen) and 52 DAE (Celle). In 2015 all females entering the cavities were counted for three mins at each nesting unit. The assessments started on 3 DAE and were conducted on every third day until the end of flowering (36 DAE at Niefern, 33 DAE at Tübingen and 36 DAE at Celle).

Additionally, the flight activity in the crop was assessed, to determine whether the bees were foraging on the crop, on three assessment dates (in 2014 on 2 DAE, 9 DAE and 15 DAE and in 2015 on 7 DAE, 14 DAE and 28 DAE) at two locations in each of the field sites (at approx. 5 m and approx. 50 m distance from the nesting units) on specified areas (4 m^2) for a specified time (3 times for 1 min in 2014, 3 times for 3 mins in 2015). In the tunnels foraging assessments were conducted three times (on days 6-7, 14-15 and 25-28) for 3 mins at each of

three areas in each tunnel (1m^2 at Niefern and Tübingen, 2m^2 at Celle). Mean values were calculated on the basis of flight activity per area per 3 min period for all assessments.

Assessment of parasitisation rate. Removal of parasites from the nesting trays started after the nesting units were transported from the field site and recorded regularly until the cocoons were transferred into cold storage for hibernation. In addition, any remaining cocoons at the end of the eclosion success assessment were opened and the number of undeveloped bees together with the number and type of parasites were counted.

Preparation of cocoons for hibernation. The nesting units were stored in a protected place under ambient conditions until full maturation of the adults in the cocoons (September). The maturation was checked by dissection of 20 randomly selected cocoons per treatment group. When full maturation was reached and the photographs for the offspring assessment were taken, cocoons removed from the nesting units (cavities). Remaining pollen and dust were sieved off and the cocoons were further cleaned by rinsing with water. After cleaning the cocoons were spread out on filter paper and dried before further processing.

Offspring production assessment. Offspring production (total viable cocoon production) was assessed, after full maturation of adult *O. bicornis* had occurred, by counting the number of viable cocoons after cleaning. The sex ratio was determined by size differentiation (male cocoons are distinctly smaller (Radmacher and Strohm 2010)), mean male cocoon weight and female cocoon weights were estimated from the total weight of the male and female cocoons produced per nest unit (8 per field, 1 per tunnel).

Eclosion success assessment. Cleaned cocoons were successively cooled down in 5 °C steps over a period of three weeks (October - November) to simulate the beginning of diapause under natural conditions. The cocoons were held at a mean temperature of 2 °C over a period of

approximately 4 months (November – February). For the assessment of eclosion success a sample of the cocoons was taken which represented 23-100% of the cocoons in 2014 and 50-100% of the cocoons in 2015. In 2014 in cases where more than 3000 cocoons were available a representative subsample was collected by taking all cocoons from every 4th tray in nesting units and in 2015 where more than 1000 cocoons were available a representative subsample was collected by taking all cocoons from alternate trays within the units.The temperature was increased stepwise from 2 °C to 10 °C and 10 °C to 22 °C over a period of 2 days, then the cocoons were incubated at approx. 22 °C (± 3 °C) to trigger eclosion of adults which occurred over a period of four weeks. Eclosion success was defined as fully developed adults emerging from incubated cocoons; any visible morphological malformations and behaviour alterations were assessed and recorded.

Offspring vigour assessment. The sex ratio was determined after eclosion of the bees; adult bees are sexually dimorphic therefore males and females were clearly distinguishable (Radmacher and Strohm 2010). The weight of the eclosed male and female bees were determined by weighing the total number of eclosed bees per field and per tunnel.

Sampling for palynology and residue analysis

Samples of nectar and pollen from the oilseed rape flowers and pollen provision from the brood cells in test unit cavities were taken and analysed for residues of thiamethoxam and its metabolite CGA322704 (common chemical name clothianidin). In addition samples of pollen provision were collected for palynology.

Winter oilseed rape pollen and nectar were collected directly from the crop from at least 12 different locations across the field (more locations were used if the same size was insufficient) at approximately 7, 14 and 21 days after the start of exposure. The pollen from

winter oilseed rape flowers was sampled by brushing pollen from the flowers into sieves with attached trays. The nectar was extracted from the flowers using a micro-centrifuge after removal of the anthers and placing the flowers in an Eppendorf vial with a fine mesh and centrifuging for less than 10 seconds at maximum speed. This was repeated until a sufficient amount of nectar was collected. A minimum of 0.2 g of pollen and nectar were collected for residue analysis and at least 30 mg nectar was also sampled for sugar content determination. The samples were frozen during transport to the laboratory (stored on dry ice) and stored deep frozen at ≤ -18 °C within a maximum of 8 hours after end of sampling. The sugar content determination was performed using a refractometer.

Samples of the pollen provision were taken directly from the brood cells in the cavities on multiple sampling occasions during flowering of the crop (weeks 1, 2 and 3 of exposure in 2014; weeks 1 to 4 of exposure in the main field in 2015; in the tunnels samples were taken in weeks 2 to 4 as no sample was available during the first week of exposure). Samples were taken randomly from at least two trays per unit. One day before sampling the position of the latest closed cell in each cavity was marked by a permanent marker on the transparent cover of the assigned trays in order to sample the pollen provision from the desired date. The pollen provision sample was collected from at least 2 different cavities of the selected trays from two different nesting units by lifting the transparent cover and using a spatula. Thus 4 replicate samples per field site were taken from the 8 nesting units and each was then divided into a sample of at least 0.2 g for residue analysis and at least 0.4 g for palynology. In the tunnels a single sample of at least 0.2 g for residue analysis was collected per tunnel at each time point. The handling time was as short as possible to avoid exposure of the shelves and the nesting units to vibrations, direct sunlight or any disturbances. The pollen provision samples were frozen during transport to

the laboratory (stored on dry ice) and stored deep frozen at ≤ -18 °C within a maximum of 8 hours after end of sampling.

Palynology

Each sample of pollen provision from open field was weighed and distilled water was added (1 part of pollen + 5 parts of water). The mixture was homogenized for one hour on a mixing plate. The sample solution (15 µL) was dispensed onto a microscopic slide and 30 µL of distilled water were added. The liquid was homogenized and spread out on a surface of 22 x 22 mm. After drying it was embedded in glyceric gelatine and covered with a cover slip. The different pollen types were identified using light microscopy at a magnification of 400 or 1000 times. The prevalence of different pollen types within 500 pollen grains per microscopic preparation were counted following DIN 10760 (DIN 2002).

Residue analysis

Crop pollen, pollen provision from brood cells and crop nectar samples were analysed using the same validated method (see Supplemental Data). Each sample was extracted using methanol/0.2% formic acid in ultrapure water (50/50, v/v), centrifuged and diluted with ultrapure water. The sample was then transferred to an HLB cartridge (60 mg, 3 mL), eluted with 6 mL acetonitrile and evaporated at 45 °C to dryness. The extract was diluted with methanol/ultrapure water (10/90, v/v) before analysis using an API 5500 QTrap LC-MS/MS (Sciex Instruments, Les Ulis, France) with Analyst 1.5.1. A Develosil RP-aqueous column (3 µm, 150 mm × 3 mm) was used with a gradient of acetonitrile and acetic acid 0.2% v/v to ultrapure water and acetic acid 0.2% v/v with a flow rate of 0.6 mL min^{-1} and a 20, 30, 50 or 100 µL injection volume.

Residues of thiamethoxam and CGA322704 in the samples analysed were calculated by alternate injections of purified specimens and external standards, using an external

standardisation procedure. Matrix-matched standards were used for analysis of both analytes in pollen and nectar sampled directly from the plants and in pollen provision from the brood cells in nest cavities. Quantification used transitions of 292.0–211.2 *m*/*z* for thiamethoxam and 249.9–169.0 *m*/*z* for CGA322704, and confirmatory transitions used were 292.0–181.1 *m*/*z* for thiamethoxam and 249.9–132.1 *m*/*z* for CGA322704. The limit of detection (LOD) and quantitation (LOQ) for both thiamethoxam and CGA322704 in pollen and for CGA322704 in nectar were 0.5 ng/g and 1.0 ng/g respectively. The LOD and LOQ for thiamethoxam in nectar were 0.25 ng/g and 0.5 ng/g respectively.

Statistical analysis

Field analysis. Analysis was conducted in SAS Proc GLM with terms in the model for location and treatment. For each endpoint the p-value for the treatment effect was reported along with the treatment means and the standard error of the difference (SED). The effect size detectable with 80% power with two sided alpha=0.05 was calculated using the central and non-central t-distribution as appropriate.

Semi-field analysis. Analysis was conducted on the tunnel level data in SAS Proc Mixed using REML, specifying the location by treatment interaction as random. For each endpoint, the p-value for the treatment effect is reported along with the treatment least square means (LSmeans) and the standard error of the difference (SED). The effect size detectable with 80% power with two sided alpha=0.05 was calculated using the central and non-central t-distribution as appropriate.

Comparison of study types. As semi-field data were only collected in 2015, the 2014 field data were excluded from this analysis. Only endpoints on a per female released basis were subjected to analysis due to the differing release rates in fields and tunnels.

Prior to analysis, the site mean of the semi-field data were calculated for the two locations which had four tunnels on each field. These data were combined with the semi-field data for the sites with only one tunnel and the field data to give a dataset with 12 data points per endpoint (three locations x two treatments x two study types). This dataset was subjected to analysis in SAS Proc GLM taking account of the split plot nature of the design (treatment as main plot and study type as subplot). For each endpoint the p-value for the study type by treatment interaction is reported.

RESULTS

Residues in pollen and nectar from crop and in Osmia *pollen provision samples*

Residue data for the crop and for pollen provisions from brood cells sampled over time from open fields and in tunnels are shown in Table 1 (detailed data are shown in the Supplemental Data). No residues of thiamethoxam or its metabolite CGA322704 (also referred to as clothianidin) were detected above the LOQ from the untreated control sites in any pollen or nectar samples from the crop or pollen provisions from brood cells. Samples taken from the treated crop across the sites contained residues in both pollen and nectar ranging from below the LOQ to a combined residue of 4 ng thiamethoxam + CGA322704/g (Table 1). The sugar content of the oilseed rape nectar varied across the fields from 29.9 ± 3.5 % to 48.0 ± 3.4 %.

Samples of *Osmia* pollen provision from brood cells in nest cavities on the open field nest sites showed similar residues to those from the treated crop ranging from below the LOQ to a combined total of 3 ng thiamethoxam + CGA322704/g. Samples of pollen provision from

nests within the tunnels showed similar residues ranging from below the LOQ to 3 ng thiamethoxam/g and up to 1 ng CGA322704/g although maximum residues of thiamethoxam and CGA322704 combined did not exceed 3 ng /g pollen provision in any sample.

Pollen sources in pollen provision samples from nests in open fields

No palynology was performed on the tunnel collected pollen provision samples as 100% oilseed rape pollen could be assumed. Palynology of the pollen provision collected from brood cells in nesting units in each field showed that between 4 % and 31 % of the pollen in cell provisions collected by the female *O.bicornis* was oilseed rape pollen (Brassica, Table 1) compared with a range of mean 36 % to 81 % *Quercus* (European oak) pollen across the 4 replicate samples and two time points per field. Other less abundant/prevalent sources of pollen included Ranunculaceae (e.g., buttercup), Rosaceae (e.g., plum), *Fagus* (beech), *Juglans* (walnut) and *Acer* (field maple). High levels of oak tree pollen were even observed in Alsace even though the nearest oak trees were more than 500m from the treated field site.

Activity at nest unit and within open fields

The flight activity at the entrance of the nesting units (number of females entering the nesting tubes) ranged from 0.7 ± 0.4 to 9.9 ± 2.6 bees per 3 min observation period in control and 3.4 ± 0.4 to 8.6 ± 2.9 bees per 3 min observation period in treated fields. Over the course of the exposure period foraging activity of the female bees within the fields 5m from the nest units was low ranging from 0.2 ± 0.3 to 1.7 ± 0.8 bees/m^2 per 3 min observation period in control and 0.4 ± 0.6 to 1.3 ± 1.1 bees/m^2 per 3 min observation period in treated fields. Activity 50 m from the nest sites was lower with no bees observed in 4 of the control and 2 treated sites and 0.6 ± 0.4 bees/m^2 per 3 min observation period in the control and 0.6 ± 0.3 bees/m^2 per 3 min observation period in the treated fields.

Activity at nest unit and within tunnels

The flight activity at the entrance of the nesting units (number of females entering the nesting tubes) was comparable between control and thiamethoxam treated tunnels ranging from 1.7 ± 2.0 to 5.4 ± 5.7 bees per 3 min observation period in control and 4.3 ± 4.9 to 7.9 ± 6.6 bees per 3 min observation period in treated tunnels.

Over the course of the exposure period foraging activity of the female bees was 2.5 ± 2.7 to 5.1 ± 3.5 bees/m^2 per 3 min observation period in control and 2.9 ± 2.6 to 6.8 ± 4.1 bees/m^2 per 3 min observation period in treated tunnels.

Reproductive parameters in open field nest sites

The number of females which eclosed from the original cocoons placed at the nest sites (not exposed to the treatment) are shown in Table 3. The number of male and female cocoons placed at each open field site varied between years and the eclosion rate was highly variable, likely related to initial cold temperatures at some field sites in 2014, ranging from 23 to 95 % in 2014 and 87.5 to 93.8 % in 2015 for females and from 37.7 to 91.2 % in 2014 and 87.9 to 93.9 % in 2015 for males. Therefore, to take account of these pre-exposure differences, reproductive parameters were divided by the number of eclosed females per field (i.e., results were expressed as number per female per field). This ensured that effects could be ascribed to treatment rather than to the initial numbers of females released.

The observed maximum nest occupation (maximum number of females observed at the nests over the course of the flowering period) represented 37.7 to 105 % (mean 73.4 %) of the number of females released in control fields, and 50.4 to 88.1 % (mean 70.2 %) of the number of females released in treated fields and reflected the release rate per field (Pearson correlation=0.88, $p<0.001$). There were no significant differences in numbers of nests completed

per released female in the control and treated fields ($F_{1,5}$=0.317, p=0.598), in the number of cells per nest ($F_{1,5}$=0.203, p=0.672); the number of female cocoons produced per female released ($F_{1,5}$=0.309, p=0.602), the eclosion success overall ($F_{1,5}$=0.000, p=0.995) or of male or female adults in 2015 ($F_{1,2}$=0.524, p=0.544 and $F_{1,2}$=0.043, p=0.855), the weights of male and female cocoons ($F_{1,5}$=0.106, p=0.758 and $F_{1,5}$=0.176, p=0.692) or of the first-generation eclosed adult males and females ($F_{1,5}$=0.450, p=0.532 and $F_{1,5}$=0.042, p=0.846) (Table 3).

The numbers of adults produced per field reflected the total numbers of nest cells and cocoons within each field site. There were no significant differences in the loss from nest cells to cocoons ($F_{1,5}$=0.559, p=0.488) and subsequent eclosion ($F_{1,5}$=0.000, p=0.995) across treated and control fields (Figure 1). Parasitisation rates of cocoons were also not significantly different across the treatments ranging from 2.0 to 28.7% at control sites and 1.4 to 28.6% at treated sites ($F_{1,5}$=0.019, p=0.896). There were no behavioural or morphological abnormalities observed in the eclosed adults.

Semi-field reproductive parameters

The eclosion rate of the original cocoons placed in the tunnels in 2015 (not exposed to the treatment) ranged from 83.3 to 100 % for females and 85.6 to 96.7 % in males resulting in the release of 50-60 females and 77-87 males per tunnel. Therefore reproductive parameters were divided by the number of eclosed females in each tunnel as for the open field released females. The observed nest occupation (maximum number of females observed at the nests) was a mean of 72 % of eclosed females across all control tunnels and 69 % across all treated tunnels. The number of nest holes filled ranged from 56 ± 2 % to 85 % of those available (Table 4). This resulted in no significant differences in the numbers of: nests completed per female released in control and treated tunnels ($F_{1,2}$=1.387, p=0.360); number of cells per nest ($F_{1,2}$=1.692, p=0.323);

number of cocoons produced per female released ($F_{1,2}$=0.296, p=0.641); overall eclosion success ($F_{1,2}$=0.940, p=0.435) or of males ($F_{1,2}$=1.520, p=0.343) and females ($F_{1,2}$=1.915, p=0.301); or weight of male and female cocoons ($F_{1,2}$=0.043, p=0.854 for males and $F_{1,2}$=1.999, p=0.293 for females) and adults ($F_{1,2}$=3.382, p=0.207 for males and $F_{1,2}$=0.714, p=0.487 for females) across the two treatments (Table 4). The numbers of adults produced per tunnel reflected the total numbers of nest cells and cocoons produced with no significant differences in rates of loss from nest cells to cocoons and subsequent eclosion across treated and control tunnels (Figure 1) with similar eclosion success of both males and females (Table 4). There were no behavioural or morphological abnormalities observed in eclosed adults. The effects of confinement were assessed by directly comparing the data generated for the tunnels with those for the same fields in 2015 in Table 5. This showed that although the number of nests per female released were not significantly affected other reproduction parameters at the level of the cells (cells per female, cells per nest, cells per nest cavity, cocoons per female) were significantly reduced in the tunnels compared with the fields on which they were placed.

Parasitisation rates of cocoons were also low in the tunnels across the sites with no parasites in sampled cocoons from treated and control tunnels at Celle and Niefern and treated tunnels at Tübingen; parasites were detected in 0.5 ± 0.9 % of sampled cocoons from the control tunnel at Tübingen.

DISCUSSION

Field and semi-field (tunnel) approaches detected no significant effects on the reproductive success of the solitary bee, *O. bicornis*, foraging on winter oilseed rape grown from thiamethoxam-treated seed. These data can also be used to understand the importance of oilseed

rape as a source of forage and develop further the design and interpretation of pesticide semi-field and field studies with *O bicornis*.

Oilseed rape as a forage source

Oilseed rape is an important crop globally with the primary production in China, India, Canada and the European Union (Carre and Pouzet 2014). In some regions neonicotinoid seed treatments have been the product of choice for early season pest control (Douglas and Tooker 2015; Hurley and Mitchell 2014), although currently imidacloprid, clothiandin and thiamethoxam are the subject of a ban in the EU on bee-attractive crops (Hurley and Mitchell 2014; Kathage et al. 2017). Although pure oilseed rape pollen has been shown to support higher offspring production than some other pollen sources, such as *Borago officinalis* (borage) or *Centaurea cyanus* (cornflower) (Bukovinszky et al. 2017), in agreement with previous studies (Holzschuh et al. 2013; Peters et al. 2016; Raw 1974; Rundlof et al. 2015), oilseed rape contributed only 4-22% of pollen provisions in this study. However, oilseed rape in the landscape has also been observed to result in increased nest building, probably due to its provision of an abundant nectar source rather than as a pollen source (Holzschuh et al. 2013; Jauker et al. 2012; Radmacher and Strohm 2010). This hypothesis that oilseed rape provides an abundant nectar source for *O. bicornis* rather than a preferred pollen source (Holzschuh et al. 2013; Jauker et al. 2012; Radmacher and Strohm 2010) is supported in this study. There were a higher number of cells per nest for free-foraging bees, which foraged over a greater distance for alternative pollen sources, than those confined to foraging on oilseed rape within tunnels in the same fields (Table 4).

Effects of confinement

Despite this lower preference for oilseed rape pollen and natural foraging range of several hundred metres (Seidelmann et al. 2010), confinement in a tunnel with approximately 1 m^2 oilseed rape plants in flower/female bee appears to have only minor effects on the reproductive behaviour. The number of nests constructed per female were similar to those in the open fields in the present study (Table 4) and to numbers in both smaller tunnels (Bukovinszky et al. 2017) and under laboratory conditions with *ad libitum* pollen and nectar (Sandrock et al. 2014). However, the average number of cells per nest observed in the tunnels were lower than those in the open fields at the same sites in 2015 ($F_{1,4}$=91.687 p=0.001), although comparable with previous reported values across a range of habitats (Fliszkiewicz et al. 2015; Jauker et al. 2012; Seidelmann et al. 2010). The natural mortality rate from eggs to eclosion was also similar to previously reported mortality rates under controlled ("ideal") laboratory conditions (Konrad et al. 2009; Radmacher and Strohm 2010). The reproduction co-efficient (number of released females to number of female cocoons) for the control and treated populations in the tunnels were both close to 2-fold which was lower than that observed in the open field (4 to 5 fold) but within the range observed in the field across a range of habitats (Fliszkiewicz et al. 2015). The available forage within the tunnels was likely not a limiting factor based on 80 plants/m^2, average flower and pollen production (Hoyle and Cresswell 2009; Pierre et al. 2003) being sufficient to produce 75 pollen brood cell provisions per m^2 (Seidelmann 2006) compared with actual production of less than 10 cells per female. Therefore, the use of large tunnels to confine *O. bicornis* on flowering oilseed rape can be considered to provide acceptable worst-case conditions whilst not severely affecting reproductive output.

The data from this study (six field sites each with one treated and one untreated field; three sites for semi-field two sites with four tunnels and one site with one tunnel) can also be

used to inform the design of future studies. A small difference (Brock et al. 2015) could have been detected in numbers of cells per nest cavity and the weight of emerged adults whereas the total numbers of cells and cocoons produced per released female was more variable (Table 6). Clearly it is important to relate the selected parameters to population changes and protection goals in defining the power required from such studies, such information is currently limited.

Residues in nectar, pollen and pollen provisions

There were no detectable residues of thiamethoxam, or its metabolite CGA322704, in the pollen and nectar collected from the control crops or pollen provisions from control nest sites and thus no potential for exposure of developing larvae. Residues were detected in the pollen and nectar from the treated crops and within the pollen provision in the nest cells (up to 3 to 4 ng thiamethoxam and CGA322704/g pollen, > 0.25 to <0.5 ng thiamethoxam and CGA322704/g nectar). Together with observations of foraging activity within the crop, this clearly demonstrates the bees were exposed to residues of thiamethoxam and CGA322704 on the treated fields and within the tunnels. The residues detected in the pollen and nectar of the treated crop were within the range of those previously reported for winter oilseed rape grown from seed treated with thiamethoxam (Figure 2) (Balfour et al. 2017; Blacquiere et al. 2012; European Food Safety 2013; Godfray et al. 2014; Godfray et al. 2015; Pilling et al. 2013): Higher residues have been reported but these were oven-dried pollen samples (Botias et al. 2016) and conversion to a wet weight basis (Firon et al. 2012; Franchi et al. 2011; Pacini et al. 2006) results in values similar to those previously reported. Both the maximum values and the variability of the residues of thiamethoxam and CGA322704 in the cell provisions reflected those measured in pollen and nectar sampled directly from the crop. This appears unexpected given the low contribution of oilseed rape pollen (<20%) to the brood cell provisions in the field. However, it

may be explained by the combined effects of evaporation/dehydration of nectar from 70% water to 20% water and the combination of 2 parts nectar to 1 part pollen during construction of the nest provision (Bosch and Vicens 2002). Effects of evaporation/dehydration and subsequent combining of nectar and pollen may also explain higher residues in provisions from other wild bee species (David et al. 2016; Woodcock et al. 2017).

Effects of neonicotinoids

There have been extensive discussions over the potential effects of neonicotinoids on wild bees and solitary bees foraging on oilseed rape (Balfour et al. 2017; Garratt et al. 2014; Nicholls et al. 2017; Woodcock et al. 2016b). Despite this there are few data available on effects under realistic conditions, in part due to the challenges of working with wild bee species in the field. Contrasting results have been reported from field studies with *O. bicornis* in oilseed rape grown from neonicotinoid treated seed. Rundlof et al. (2015) reported *O. bicornis* failed to nest in sites in fields of spring oilseed rape grown from clothiandin-treated seed; Woodcock et al. (2017) reported lower levels of cell production in oilseed rape fields where samples of nest pollen provision containing higher median, but not peak levels, of total neonicotinoids; whereas Peters et al. (2016) reported no effects following exposure to winter oilseed rape grown from clothiandin-treated seed. However, the design of these studies varied. Rundlof et al. (2015) released an average of 5 females in 8 Ha fields (only 40% of the 12 female cocoons eclosed per field), Woodcock et al. (2017) placed 25 females cocoons per field of an average 40 Ha, but did not report eclosion rates or actual numbers of females observed at nests (in addition only a subset of the nest sites were evaluated), Peters et al. (2016) released an average of 680 females per 35 Ha field. In the present study 480 to 1200 females were released in 1.9 to 2.7 Ha fields.

Losses are likely to occur from eclosion to nest founding; the number of released females actually observed at the nest sites in the present study (mean across all fields was 69%; across all tunnels was 70%) were comparable to those reported by Peters et al. (2016) (mean 65%). The numbers of females observed at nest sites were not reported by Rundlof et al. (2015) but, based on a 30-35% loss, an average of only 3-4 females per field would be expected to build nests, but this may be an underestimation of the actual loss.. Based on the expectation that one female constructs 1-1.5 nests (each nest was reported to contain a mean of 3.5 ± 0.3 cells which is within the expected range) and a median of 1.4 (mean 2.9) nest tubes used per field, this suggests that in fact an average of only 1 to 2 females were present in the control fields in the Rundlof et al. (2015) study. Therefore, the reported absence of nesting females on the treated sites should be put in the context of the very small numbers of females on the control site.

Offspring sex ratio and size

Oilseed rape nectar may be important forage for *O. bicornis* particularly in fuelling flights to preferred pollen sources. Thus exposure to thiamethoxam in nectar may have consequences on individual females as maternal provisioning affects offspring sex ratio with lower provisioning resulting in a shift to male production (Radmacher and Strohm 2010). A single laboratory study (Sandrock et al. 2014) reported a significant effect of nectar containing thiamethoxam/clothianidin on the number of nests per *O. bicornis* female (22% reduction) and number of cells per nest (33% reduction) resulting in an overall reduction in reproductive output of close to 50% with a significant bias to male production. A similar shift to male production was noted by Peters et al.(2016) with oilseed rape grown from clothianidin treated seed, with the caveat that nearly 50% more females were produced in total at the treated site compared to the

control site. However, in the present study no treatment-related reduction in total cocoon production or in production of female cocoons was observed in the fields or tunnels.

Provision size within the nest cell is one of the most important factors determining offspring body size (Radmacher and Strohm 2010; Roulston et al. 2000) which is related to fitness in solitary bees through enhanced foraging efficiency (Torchio and Tepedino 1980), fecundity (Kim 1997) and overwintering survival (Bosch and Kemp 2004). Any effects of pesticide residues on consumption of nest provisions by larvae would thus be expected to be reflected in cocoon and adult body weight. In line with a single laboratory study in which *O. bicornis* larvae were directly fed with 10 ng clothiandin/g pollen (Nicholls et al. 2017) there was no effect of treatment on mean cocoon or adult weights in this study and they were within normal ranges (Radmacher and Strohm 2010; Sandrock et al. 2014).

Parasitisation

The level of cell parasitisation has been suggested to reflect the ability of females to locate and provision nest sites (Artz and Pitts-Singer 2015; Jauker et al. 2012) as parasites enter the nest cells when the female is absent from the nest; longer provisioning times may thus result in increased incidence of parasitisation. Levels of parasitisation in the open field were comparable between treatment and control and to those previously reported (Fliszkiewicz et al. 2015; Jauker et al. 2012; Peters et al. 2016; Seidelmann 1999) but higher than those in the tunnels. The close proximity of the source of pollen to the artificial nest sites within the tunnels likely reduced the foraging time and thus the time available for parasites to enter the cells.

CONCLUSIONS

The present study has shown no detectable effects on the reproductive output of *O. bicornis* nesting within fields or tunnels of winter oilseed rape grown from thiamethoxam-treated

seed. Although crops such as oilseed rape may be an important source of nectar, other sources of pollen for females provisioning their nests in the field were preferred. The present study has also shown the challenges of working with free-flying *Osmia*. Reproductive performance in the field should not rely solely on total numbers of cells/cocoons/adults but take into account the number of females released and successful development of cells.

Whilst the use of semi-field (tunnel) studies may provide worst-case conditions with more opportunity for replication than in the open field, realism is limited by the lack of alternative forage. For field studies with pesticides with short half-lives in pollen and nectar a pre/post application comparison of nest construction with females with established nest sites may be considered more appropriate. However, limitations of the field scenario include crop attractiveness and, in agreement with a number of other studies, good quality habitat is important in determining wild bee populations and their development through the provision of forage sources.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx

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Data availability—Data, associated metadata, and calculation tools are available from the corresponding author (Natalie.Ruddle@syngenta.com).

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Figure 1. Summary of cocoon production and eclosion success rates for control and treated sites under open field conditions in 2014 and 2015.

Figure 2. Comparison of total residues of thiamethoxam and CGA322704 in pollen collected from the crop and Osmia pollen provisions with crop pollen data from Pilling et al (2013).

Table 1 Contribution of Brassica (oilseed rape) and *Quercus* (oak) pollen grains and residue analysis results from *Osmia bicornis* collected pollen masses and for crop pollen and nectar collected in the control and treated oilseed rape fields at each of the 6 sites and tunnels at 3 sites in 2015

	Control fields						Treated fields					
	% open field nest pollen mass (mean ± SE)		Range of residues thiamethoxam + CGA322704 (ng/g)				% open field nest pollen mass (mean ± SE)		Range of residues thiamethoxam + CGA322704 (ng/g)			
Site	Brassica	*Quercus*	crop pollen	crop nectar	pollen mass from open field	pollen mass from tunnel	Brassica	*Quercus*	crop pollen	crop nectar	pollen mass from open field	pollen mass from tunnel
2014												
Alsace, France	19 ± 3	59 ± 5	<LOD	<LOD	<LOD	-	22 ± 4	69 ± 3	<LOD	<LOD	<LOD	-
Tübingen, Germany	14 ± 2	81 ± 2	<LOD-<LOQ	<LOD	<LOD	-	7 ± 2	86 ± 2	<LOD-<LOQ	<LOD	<LOD	-
Kraitchal, Germany	31 ± 2	53 ± 8	<LOD	<LOD	<LOD	-	21 ± 2	77 ± 2	<LOD	<LOD-<LOQ	<LOD	-
2015												
Celle, Germany	22 ± 2	58 ± 9	<LOQ	<LOQ	<LOQ	<LOQ	31 ± 7	64 ± 8	3-4	<LOQ	<LOQ-3	<LOQ-3
Tübingen, Germany	10 ± 2	58 ± 8	<LOQ	<LOQ	<LOQ	<LOQ	5 ± 1	69 ± 8	<LOQ-1	<LOQ	<LOQ-2	<LOQ-1
Niefern, Germany	4 ± 1	67 ± 4	<LOQ	<LOQ	<LOQ	<LOQ	3 ± 1	36 ± 9	<LOQ-4	<LOQ	<LOQ	<LOQ

2014: LOD 0.25 ng thiamethoxam /g nectar; 0.5 ng/g CGA322704/g nectar; 0.5ng thiamethoxam/g pollen; 0.5 ng CGA322704 /g pollen

2014/2015: LOQ: 0.5 ng thiamethoxam /g nectar; 1.0 ng/g CGA322704/g nectar; 1 ng thiamethoxam /g pollen; 1 ng CGA322704 /g pollen

Table 2 Location of sources of *Quercus* (oak) pollen at each open field site (for aerial photos showing proximity of woodland see supplementary information)

Site	Proximity of oak trees at control field	Proximity of oak trees at treated field
Alsace, France, 2014	present in the surroundings of field site in distances between approx. 190- 270 m (mean distance 230 m), medium abundance	present in the surroundings of field site in distances between approx. 540-1500 m (mean distance 1020 m), low abundance
Tübingen, Germany, 2014	present in the surroundings of field site in distances between approx. 160-330 m, medium abundance	present in the surroundings of field site in distances up to 220 m, high abundance
Kraitchal, Germany, 2014	present in the surroundings of field site in distances between approx. 199-280 m	present in the surroundings of field site in distances of 35-320 m
Celle, Germany, 2015	present next to the field site (within 100m)	present next to the field site (within 100m)
Tübingen, Germany, 2015	present in surrounding mixed forest near field site (within 100 -200 m)	present in mixed forest near field site (within 100 -200 m)
Niefern, Germany, 2015	many in deciduous forest next to the field site (within 100m)	few in deciduous forest next to the field site (within 100m)

Table 3 Reproductive parameters for *Osmia bicornis* in the open oilseed rape fields for each site and treatment (control and thiamethoxam seed treatment) (mean (± SE are shown for fields where nest parameters are attributed to the 8 replicate nest sites))

	2014 Alsace		2014 Tübingen		2014 Kraitchal		2015 Celle		2015 Tübingen		2015 Niefern		Control vs treated p=
Parameter	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	Control	Treated	
Females released	864	1101	383	270	1138	288	440	420	444	434	450	438	-
Nest cavities available per female released	2.8	2.2	6.3	8.9	2.1	8.3	1.8	1.9	1.8	1.8	1.8	1.8	-
% nest cavities used (total available 2014: 2400, 2015: 800)	59	36	18	16	30	20	31	88	99	81	99	99	-
Females observed at nest	621	555	190	165	731	199	166	370	366	332	474	333	0.362
Total nests	1408	859	425	394	1275	490	247	703	793	648	790	792	0.371
Nests/female released	1.63	0.78	1.11	1.46	1.12	1.70	0.56	1.67	1.79	1.58	1.77	1.82	0.598
Total cells produced	15062	5512	2878	2376	9728	4398	1357	5045	6529	4854	6358	6599	0.300
Cells/nest	10.1 ± 0.5	6.4 ± 0.2	5.9 ± 0.6	6.5 ± 0.4	7.5 ± 0.3	7.9 ± 0.7	4.9 ± 0.6	7.0 ± 0.5	8.2 ± 0.2	7.1 ± 0.5	8.0 ± 0.4	8.3 ± 0.3	0.672
Total cocoons	12449	4180	1705	1223	9029	2032	1140	4855	5446	4622	5948	6026	0.306
Female cocoons/female released	8.6	2.6	2.1	2.1	4.5	3.9	1.1	4.8	4.7	4.4	6.6	5.7	0.602
Female cocoon weight (g)	0.115 ± 0.003	0.099 ± 0.002	0.111 ± 0.010	0.097 ± 0.004	0.099 ± 0.001	0.093 ± 0.005	0.115 ± 0.002	0.122 ± 0.004	0.097 ± 0.004	0.114 ± 0.003	0.103 ± 0.004	0.102 ± 0.002	0.692

Male cocoon weight (g)	0.064 ± 0.002	0.056 ± 0.001	0.056 ± 0.005	0.063 ± 0.007	0.059 ± 0.001	0.053 ± 0.002	0.065 ± 0.002	0.074 ± 0.002	0.061 ± 0.001	0.068 ± 0.002	0.066 ± 0.001	0.063 ± 0.001	0.758
Eclosion success females (%)	79.8	74.3	58.6	68.7	84.5	86.5	86.7	86.5	80.6	85.7	86.2	79.1	0.855
Eclosion success males (%)							83.4	86.1	86.1	88.2	87.0	86.3	0.544
Female adult weight (g)	0.089 ± 0.001	0.087 ± 0.001	0.080 ± 0.001	0.070 ± 0.005	0.080 ± 0.002	0.080 ± 0.004	0.086 ± 0.007	0.096 ± 0.001	0.078 ± 0.016	0.088 ± 0.001	0.083 ± 0.001	0.079 ± 0.001	0.846
Male adult weight (g)	0.050 ± 0.001	0.050 ± 0.0004	0.042 ± 0.001	0.041 ± 0.001	0.047 ± 0.001	0.045 ± 0.001	0.049 ± 0.001	0.056 ± 0.002	0.045 ± 0.001	0.051 ± 0.001	0.049 ± 0.001	0.046 ± 0.0004	0.532

Table 4 Reproductive parameters by site and treatment for tunnels on the fields in 2015 (mean ± SE) Data were normalised to the number of females released per tunnel.

	Celle (N=3)		Tübingen (N=3)		Niefern (N=1)		
Parameter	Control	Treated	Control	Treated	Control	Treated	P value
Females released	53.5 ± 0.5	54.8 ± 0.3	55.3 ± 1.9	53.5 ± 0.9	56	60	-
Females observed at nest	37.8 ± 4.3	34.3 ± 0.9	39.3 ± 3.1	41.0 ± 3.4	43	40	-
% Nest holes used per tunnel (of 100)	58 ± 10	56 ± 2	76 ±5	69 ± 5	83	85	-
Nests/female	1.07 ± 0.08	1.02 ± 0.02	1.38 ±0.09	1.3 ± 0.06	1.48	1.42	0.360
Total cells/tunnel	191.3 ± 15.6	228.5 ± 9.2	405.8 ± 19.3	359.0 ± 12.2	343	434	0.633
Cells/nest	3.3 ± 0.1	4.1 ± 0.3	5.4 ± 0.6	5.2 ± 0.4	4.1	5.1	0.323
Total cocoons/female	3.22 ± 0.34	3.75 ± 0.08	7.04 ± 0.42	6.5 ± 0.27	5.73	7.02	0.641
Female cocoons/female	1.39 ± 0.08	1.60 ± 0.09	2.81 ± 0.27	2.90 ± 0.09	2.39	3.12	0.273
Female cocoon weight (g)	0.115 ± 0.007	0.111 ± 0.003	0.133 ± 0.006	0.108 ± 0.012	0.112	0.105	0.293
Male cocoon weight (g)	0.067 ± 0.003	0.069 ± 0.001	0.072 ± 0.002	0.069 ± 0.002	0.069	0.072	0.854
Eclosion success females (%)	90.7 ± 1.1	85.9 ± 6.1	87.5 ± 1.9	64.9 ± 12.9	72.1	78.8	0.301
Eclosion success males (%)	88.1 ± 3.8	87.3 ± 3.0	87.0 ± 3.3	79.6 ± 6.1	81.4	71.5	0.343
Female adult weight (g)	0.094 ± 0.003	0.093 ± 0.002	0.089 ± 0.002	0.092 ± 0.005	0.094	0.074	0.487
Male adult weight (g)	0.050 ± 0.002	0.052 ± 0.001	0.050 ± 0.004	0.051 ± 0.001	0.049	0.052	0.207

Table 5 Comparison of reproductive parameters generated in the open fields and tunnels in 2015

Parameter	Comparison of study type (field vs tunnels) p value	Direction of Change
Total nests per female released	0.203	Field = tunnel
Total cells per released female	0.017	Field >tunnel
Cells per nest cavity	0.001	Field >tunnel
Cocoons per released female	0.018	Field >tunnel
Female cocoons per female released	0.035	Field >tunnel
Eclosion success females	0.427	Field = tunnel
Eclosion success males	0.192	Field = tunnel

Table 6 Summary table of relative minimum detectable differences (MDD) for various measures of *O. bicornis* reproduction and development under field and tunnel conditions

Parameter	Field: Detectable effect size (%) *	Tunnel: detectable effect size (%) *
Total nests	77%	22%
Total cells	96%	70%
Total nests per female released	74%	26%
Total cells per released female	101%	51%
Cells per nest cavity	40%	47%
Cocoons per released female	98%	51%
Eclosion success	12%	32%
Cells to cocoons	38%	14%
Weight male cocoon	18%	16%
Weight female cocoon	17%	17%
Weight adult male	14%	11%
Weight adult female	14%	42%

*At 80% power, alpha=0.05

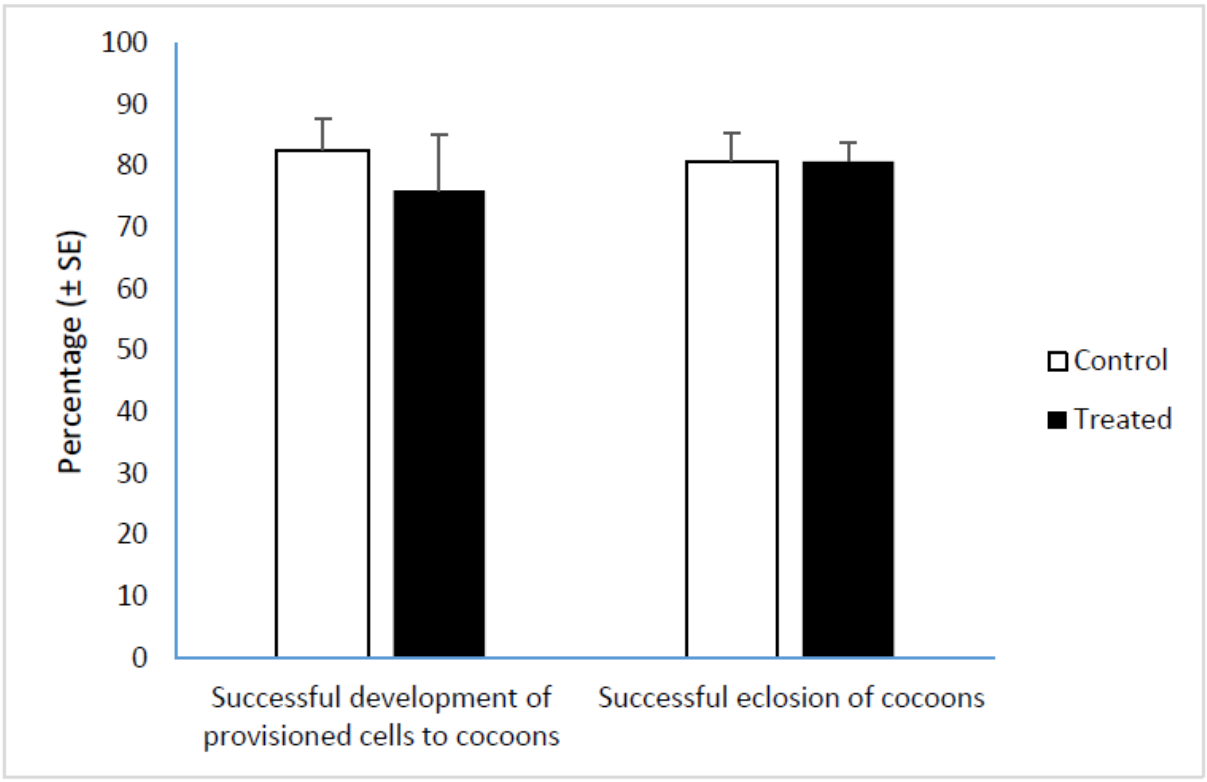

Figure 1

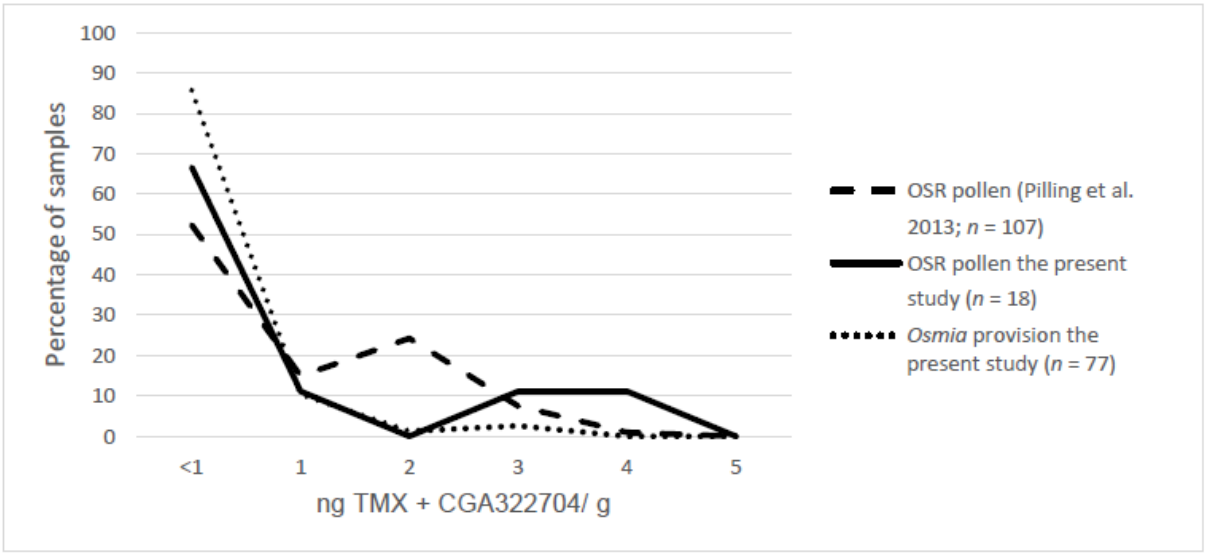

Figure 2