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Influence of combined pesticide and parasite exposure on bumblebee colony traits in the laboratory

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Summary

1. Pollinating insects provide vital ecosystem services of enormous importance for economies and biodiversity. Yet, there is a concerning global trend of pollinator declines. Parasites and pesticides are among the suspected principle drivers of these declines. However, especially in the case of key wild pollinators, there are insufficient data on the relative impact of these individual environmental stressors and whether they interact to increase detrimental effects.

2. Using a fully crossed factorial design, we investigated how laboratory exposure to neonicotinoid insecticides, thiamethoxam and clothianidin, over a 9-week period and a prevalent trypanosome gut parasite *Crithidia bombi* affects various crucial colony traits of the bumblebee *Bombus terrestris*.

3. We show that chronic dietary exposure from an early stage of colony development to doses of thiamethoxam and clothianidin that could be encountered in the field truncated worker production, reduced worker longevity and decreased overall colony reproductive success. Further, we demonstrate a significant interaction between neonicotinoid exposure and parasite infection on mother queen survival. The fate of the mother queen is intrinsically linked to colony success, and under combined pressure of parasite infection and neonicotinoid exposure, mother queen survival was lowest. This indicates increased detrimental effects of combined exposure on this crucial colony trait. Combined effects may be exacerbated in stressful natural environments where more pronounced parasite virulence is expected.

4. *Synthesis and applications*. Our findings reiterate that dietary exposure to neonicotinoids can impact on bumblebee colony performance and fitness. The indication of combined negative effects of ecologically relevant pressures suggests additional adverse consequences for long-term population dynamics under complex field conditions. To help safeguard pollinator health, whole life-cycle fitness assessments, particularly for non-*Apis* bees, stringently incorporating chronic and sublethal side effects of pesticides, as well as interactions with common natural stressors, such as prevalent parasites, should be considered in the corresponding test guidelines.

Key-words: *Bombus*, *Crithidia*, environmental interactions, fitness, neonicotinoid, pesticide risk assessment, pollinator, reproductive success, sublethal effect

Introduction

The maintenance of food security and biodiversity constitutes key challenges for modern human societies. Insect pollination services are particularly important for safeguarding agricultural productivity (Klein *et al.* 2007; Aizen *et al.* 2009; Garibaldi *et al.* 2011a) and ecosystem stability (Bascompte, Jordano & Olesen 2006; Fontaine *et al.* 2006). There is growing awareness about the economic and ecological value of pollinators, including the future prospect of vastly increased demands for the services that they provide (Klein *et al.* 2007; Aizen *et al.* 2008; Gallai *et al.* 2009). In this context, mounting evidence for global pollinator declines over the last decades (Biesmeijer *et al.* 2006; Goulson, Lye & Darvill 2008; Potts *et al.* 2010; Cameron *et al.* 2011) is alarming (although see Carvalheiro *et al.* 2013).

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Intriguingly, world-wide declines of various pollinator insects with diverse life histories and environmental requirements suggest the involvement of common causal factors. Parasites and pathogens and widely occurring fragmentations of natural habitats through land-use intensification are suspected drivers (Goulson, Lye & Darvill 2008; Potts *et al*. 2010; Cameron *et al*. 2011; Garibaldi *et al*. 2011b; Nazzi *et al*. 2012). Threats to pollinators throughout agricultural areas are also imposed by the widespread use of pesticides in crop protection (Desneux, Decourtye & Delpuech 2007). Systemic pesticides such as neonicotinoid insecticides are particularly problematic because they can result in chronic dietary exposure through trace residues in the pollen and nectar of treated plants (Cresswell, Desneux & vanEngelsdorp 2012). Over the last decade, neonicotinoid applications have substantially expanded on many crops world-wide (Elbert *et al*. 2008; Jeschke *et al*. 2011). Neonicotinoids are very efficient in combating insect pests, acting as specific agonists of the insect acetylcholine receptors and disrupting neuromuscular signalling pathways (Elbert *et al*. 2008; Jeschke *et al*. 2011). Nevertheless, non-target organisms such as pollinators can also be affected by ingesting neonicotinoid-contaminated pollen and nectar (Desneux, Decourtye & Delpuech 2007; Cresswell 2011; Blacquière *et al*. 2012). In honeybees, for example, sublethal dietary exposure to neonicotinoids was shown to negatively impact foraging, homing behaviour and cognitive and learning abilities (Decourtye & Devillers 2010; Belzunces, Tchamitchan & Brunet 2012; Blacquière *et al*. 2012; Henry *et al*. 2012). In addition, there is accumulating evidence, mostly from laboratory studies, that combined exposure to routinely used neonicotinoids and common parasites can exacerbate detrimental effects in honeybees (Alaux *et al*. 2010; Vidau *et al*. 2011; Pettis *et al*. 2012).

The honeybee *Apis mellifera* generally serves as a surrogate for pollinating insects in pesticide hazard evaluations (OECD 1998a,b; OEPP/EPPO 2010a,b), although it remains unclear whether specific responses can be extrapolated to other pollinators with contrasting life histories (Desneux, Decourtye & Delpuech 2007; Goulson, Lye & Darvill 2008; Mommaerts *et al*. 2010). As opposed to perennial honeybee colonies that comprise many thousands of workers, in comparably small-sized annual bumblebee colonies, there is a more direct link between individual performance and overall fitness. Therefore, they could suffer more from sublethal detrimental impacts of pesticides (Osborne 2012). Indeed, while conclusive inferences of durable effects of neonicotinoids on honeybee colonies are rarely compelling (Cresswell 2011; Cresswell, Desneux & vanEngelsdorp 2012), there is indication for negative fitness effects in bumblebees (Goulson, Lye & Darvill 2008). Recently, Whitehorn *et al*. (2012) and Larson, Redmond & Potter (2013) demonstrated that field-realistic chronic exposure to the neonicotinoids imidacloprid and clothianidin, respectively, significantly decreased colony growth rates and reduced daughter queen production by 85% or more. These findings were recently supported and potentially explained mechanistically by Gill, Ramos-Rodriguez & Raine (2012), although compared to Whitehorn *et al*. (2012) actual imidacloprid concentrations in the food supplements administered to otherwise freely foraging colonies were in the upper range of environmentally relevant residue levels. Gill, Ramos-Rodriguez & Raine (2012) showed that colony provisioning efficiency is significantly impaired upon chronic exposure to imidacloprid. Moreover, the results of these semi-field studies are in line with comparable laboratory experiments revealing adverse effects on foraging behaviour and a reduction in bumblebee worker fecundity in microcolonies by 42–60% (Mommaerts *et al*. 2010; Laycock *et al*. 2012). These insights are indicative of covert side effects of sublethal neonicotinoid exposure being most likely expressed when traded off against costly pollinator life-history investments and could translate to reduced reproductive investment in natural environments (Whitehorn *et al*. 2012; Larson, Redmond & Potter 2013). Therefore, it is surprising that lifetime reproductive success of Apoidean pollinators has received little attention to pesticide risk assessment (Desneux, Decourtye & Delpuech 2007). A better understanding of sublethal pesticide effects on pollinator fitness and potential long-term impacts on population dynamics is critically needed, including more detailed explorations of key non-*Apis* pollinator systems. Furthermore, given exposure to multiple environmental stressors in the field, it is vital that studies assess how pesticides may interact detrimentally with other major antagonistic factors, including natural parasites.

To contribute to the knowledge base in the ongoing debate on whether widely applied systemic insecticides have negative impacts on pollinators, we present a fully crossed factorial experiment addressing the individual and combined effects of sublethal neonicotinoid exposure and parasite infections on important colony traits of the bumblebee *Bombus terrestris*. For the neonicotinoid treatment, we applied thiamethoxam and its major bioactive metabolite clothianidin in pollen and nectar substitute provisions. A mixed diet of these two compounds was provided as both neonicotinoids will principally co-occur in nectar and pollen of crops treated with plant protection products containing thiamethoxam (Nauen *et al*. 2003; Dively & Kamel 2012; Pohorecka *et al*. 2012) and thus jointly be encountered by foraging pollinators. Thiamethoxam is the second most important neonicotinoid after imidacloprid, in terms of sales and crop usage, and is widely used for systemic protection in agroecosystems (Elbert *et al*. 2008). For the parasite challenge, we used controlled infections of the common trypanosome gut parasite *Crithidia bombi*, which has previously been shown to have a strong condition-dependent impact on bumblebee fitness (Schmid-Hempel 2001; Brown, Schmid-Hempel & Schmid-Hempel 2003). Concurring with recent reports on imidacloprid (Gill, Ramos-Rodriguez & Raine 2012; Whitehorn *et al*. 2012) and clothianidin (Larson, Redmond & Potter 2013),

we demonstrate that chronic dietary exposure to thiamethoxam and clothianidin impairs bumblebee colony growth performance and reduces colony reproductive success. We further present the first evidence in bumblebees that combined neonicotinoid and parasite exposures can enhance detrimental effects on essential colony traits, notably mother queen longevity.

Material and methods

STUDY ORGANISMS: BEES AND PARASITES

The bumblebee *B. terrestris* (Linnaeus 1758; Hymenoptera: Apoidea) is one of the most abundant wild pollinators across Europe (Goulson, Lye & Darvill 2008). Bumblebees are eusocial, exhibiting annual colony cycles in temperate regions. An individual colony's reproductive success largely depends on both the mother queen's condition and the presence of a sufficiently large worker force (Müller & Schmid-Hempel 1992; Imhoof & Schmid-Hempel 1999).

The trypanosome gut parasite *C. bombi* is common across bumblebee populations, reaching the prevalence of up to 30% in spring queens and 80% in summer worker populations (Shykoff & Schmid-Hempel 1991; Gillespie 2010). It is readily transmitted within and between colonies via faeces on nest material or flowers (Schmid-Hempel 2001). While *C. bombi* rarely has negative effects on individual hosts or colonies in favourable environments, condition-dependent virulence is observed in food-stressed workers (Brown, Loosli & Schmid-Hempel 2000), and infection strongly reduces success of queens during stressful colony founding (Brown, Schmid-Hempel & Schmid-Hempel 2003). Furthermore, efficiency of foraging is reduced in infected workers (Gegear, Otterstatter & Thomson 2005, 2006).

ANIMAL REARING AND TREATMENTS

Parasite-free *B. terrestris* colonies reared from wild-caught queens originating from a single source population in northern Switzerland (47°28′31″N, 7°35′11″E) provided queens and males that were mated. These first-generation laboratory-reared queens were artificially hibernated at 4 °C for 80 days. Upon removal from hibernation, queens were kept individually in transparent plastic boxes (12·5 × 7·5 × 5·5 cm) under red light at 28 °C and 60% relative humidity. Equal numbers of sister queens within mating combinations were randomly assigned to one of four groups, in order to ensure evenly distributed genetic backgrounds. The parasite-free status of queens and their offspring was checked periodically. When colonies had produced ten workers, they were transferred to gypsum nests (30 cm diameter × 20 cm height) with a tube connected to a foraging box (*c.* 2 L volume), and the experimental regime was initiated. Sugar water (35%, with equal proportions of glucose, fructose and saccharose) and pollen patties containing two-thirds fresh honeybee pollen and one-third sugar water were provided *ad libitum*. Subsamples of the commercial sugar syrup and the mixed stock of pollen used were analysed for the residues of thiamethoxam and clothianidin using established GC–MS methods and applicable standards for both compounds with a limit of detection of up to 0·1 p.p.b. (United States Department of Agriculture Agricultural Marketing Service National Standards Laboratory, Gastonia, NC, USA). Neither compound was detected in the sugar syrup or in selected pollen sources. Pollen supplies were renewed every 72 h, and nectar substitutes were replaced once per week. Colony food collection was tracked over the course of the experiment to infer corresponding collection per bee.

The 4 groups consisting of 10 colonies each were randomly assigned to one of the following treatments of our fully crossed factorial design: (P) *C. bombi* infection only, (N) neonicotinoid (thiamethoxam and clothianidin) exposure only, (PN) combined neonicotinoid exposure and parasite infection and (C) non-challenged controls. Subsequently, we refer to these groups by the letters in brackets.

Infections of colonies with *C. bombi* were ensured as follows. At the 10-worker stage, 5 workers of each colony were isolated, kept individually and starved for 2 h. All individuals received 10 μL of sugar water to imbibe, either containing *C. bombi* cells (groups P and PN) or no supplement (groups N and C), and were subsequently reintroduced to their natal colony. *Crithidia bombi* treatments comprised 20 000 cells, with an equal mixture of four different strains previously collected from Switzerland and cultured in the laboratory (Salathé *et al.* 2012). Infection success was confirmed by collection of faeces and counting parasite cells under a microscope at 14, 28 and 43 days after regime initiation. The number of *C. bombi* cells in the faeces was counted for 4–13 randomly chosen workers from each colony across the time points. Similarly, we confirmed the absence of *C. bombi* infections in the N and C groups 14, 28 and 43 days after regime initiation and thereafter on a monthly basis.

One day after the transfers to the experimental regime and initiating of gut parasite infections, we started differential feeding treatments, that is dietary neonicotinoid exposure (N and PN groups) versus non-spiked nutrition (P and C groups). We applied thiamethoxam and its major metabolite clothianidin simultaneously over a 9-week period. Pure compounds (Fluka, analytical standard) were purchased from Sigma-Aldrich (Seelze, Germany) and dissolved in stock solutions of distilled water. On the day of provisioning, aliquots were used for spiking both nectar substitutes and pollen patties to contain concentrations of 4 μg kg^{-1} (4 p.p.b.) thiamethoxam and 1·5 μg kg^{-1} (1·5 p.p.b.) clothianidin, respectively, to simulate chronic dietary exposure in N and PN treatment groups during the colony growth period, that is between the 10-worker stage and the senescent phase. The applied concentrations correspond to environmentally relevant residue levels reported from field samples of nectar and pollen of several flowering crops such as oilseed rape, sunflower or maize systemically treated with thiamethoxam or neonicotinoids in general (Blacquière *et al.* 2012; Pohorecka *et al.* 2012), although considerably higher concentrations have also been measured, for example, in cucurbits (Dively & Kamel 2012; Stoner & Eitzer 2012). A continued exposure, as in our experiment, represents a worst-case scenario, as switching between foraging resources in the field may rather result in pulsed exposure. However, widespread pesticide use, together with non-synchronous flowering of crops, and possible non-crop contamination, for example via dust drift, mean that there is the potential for more continuous exposure to be realised in agroecosystems.

DATA COLLECTION

After experimental regime initiation, colony worker production was documented three times per week. Newly emerged workers

were individually marked in order to trace longevity. Similarly, we tracked the survival of mother queens, which were synchronised with regard to age (±48 h), and mating and hibernation dates.

Newly emerged gynes and males were monitored and removed on a daily basis. After the mother queen's death, sexuals were only counted for an additional 21 days to exclude biases by considering workers' male offspring later on. Colony fitness was estimated as the number of males produced plus two times the number of gynes, as has previously been used as a measure of investment in sexual reproduction accounting for the greater per-individual investment into gynes (Baer & Schmid-Hempel 1999).

Colony pollen and sugar water collection could be converted into collection per bee per day across different treatments because exact numbers of workers present in each colony at a given time were known. But note that inferences of daily neonicotinoid dose ingested per bee were not possible because of varying amounts of brood.

STATISTICAL ANALYSES

Weekly worker production was analysed using a repeated-measures ANOVA. A complete model was fitted with neonicotinoid and parasite exposure, and week after the initiation of the experiment as fixed factors, and the interaction between the three. Colony was included as a random effect. *Post hoc* *t*-tests on worker production were carried out individually for each week across the colony cycle.

Worker longevity was analysed using a Cox proportional hazard model. Colony was included as a random factor using a frailty term in the model. Full models were fitted with neonicotinoid exposure and parasite infection status as fixed factors, and the interaction between the two.

Colony sexual investment and production of males alone was analysed using a generalised linear model fitted with a quasi-Poisson error distribution and a log-link function to account for over-dispersion. The propensity for colonies to produce gynes was analysed using a generalised linear model with a quasi-binomial error distribution and a logit link function. Full models were fitted with pesticide exposure and parasite exposure as factors, and the interaction between the two.

A Cox proportional hazard model was used to assess the influence of neonicotinoids and parasites, and the interaction between the two on the survival of mother queens.

Pollen and sugar water collection data (amount removed from feeders), converted to per bee per week, were analysed over the 9 weeks of worker production with repeated-measures ANOVAS. A complete model was fitted with neonicotinoid and parasite exposure, and week after the initiation of the experiment as fixed factors, and the interactions between the three. Colony was included as a random effect. Pollen collection was transformed ($y^{0.5}$) to meet the assumptions of the model.

Counts of parasite cells in the faeces from 14, 28 and 43 days after experimental initiation were transformed to meet model assumptions ($(y + 0{\cdot}5)^{0.25}$) and analysed using a linear model with colony as a random factor and neonicotinoid treatment and time as fixed factors.

All analyses were performed using R (R Core Development Team 2011). For all tests, terms were only retained in the models if they significantly increased corresponding fits, and only best-fitting minimal models are reported here.

Results

WORKER PRODUCTION AND LONGEVITY

Unsurprisingly, week of the experiment had a significant effect on worker production ($F_{8,304} = 19{\cdot}54$, $P < 0{\cdot}001$), with worker production initially increasing before subsequently falling away towards the end of the colony cycle and the production of sexuals. Yet, independent of the parasite infection status, there was a significant interaction between week of the experiment and neonicotinoid exposure ($F_{8,304} = 3{\cdot}47$, $P < 0{\cdot}001$). This significant interaction comes from the fact that while peak production was similar across treatments, worker production in neonicotinoid-exposed colonies dropped more rapidly (Fig. 1). *T*-tests on worker production carried out individually for each time point between neonicotinoid-exposed (N and PN groups) and non-exposed colonies (P and C groups) revealed that while there were no differences during the first 3 weeks, the former exhibited significant (weeks 4 and 7) differences and non-significant trends of lower worker production in later weeks.

Further, there was a significant influence of neonicotinoid exposure on worker longevity (Cox proportional hazard: $\chi^2_1 = 4{\cdot}75$, $P = 0{\cdot}029$). Workers exposed to neonicotinoids had a lower survival rate compared to non-exposed workers [hazard ratio = 1·47 (lower 95% CI = 1·04; upper 95% CI = 2·07)]. Mean (SE) longevities

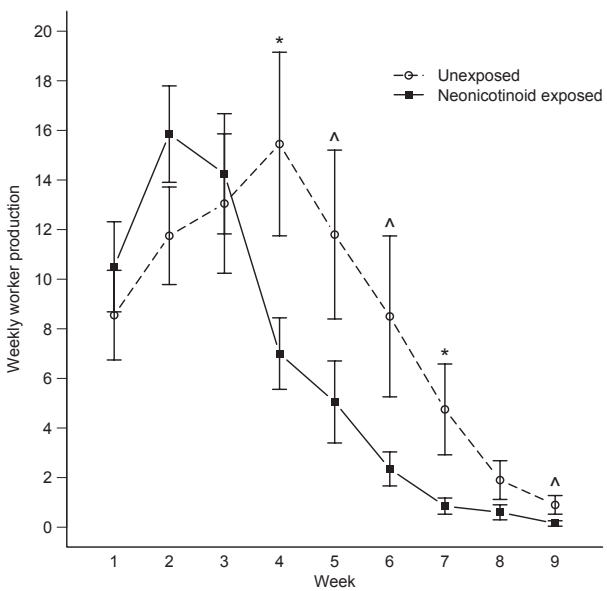

Fig. 1. Chronic dietary neonicotinoid (thiamethoxam and clothianidin) exposure and weekly production of workers of all colonies across the 9 weeks of worker production after initiation of the experiment. Independent of parasite infection (see Results), dashed lines and open symbols represent non-exposed colonies ($n = 20$), while solid lines and filled symbols represent exposed colonies ($n = 20$). Points show mean numbers (±SE) of produced workers. Symbols above bars represent the results of individual *post hoc* *t*-tests for each time point (*$P < 0{\cdot}05$; ^$0{\cdot}05 < P < 0{\cdot}1$).

for workers across colonies (19·9 workers tracked per colony on average) for C, N, P and NP treatments were 61·0 (±4·9), 54·7 (±3·1), 59·5 (±5·4) and 46·2 (±5·1) days, respectively.

SEXUAL INVESTMENT

Overall colony sexual investment was not affected by *Crithidia* infections ($F_{1,37} = 0{\cdot}22$, $P = 0{\cdot}64$). Neonicotinoid exposure, however, significantly decreased colony sexual investment ($F_{1,38} = 5{\cdot}78$, $P = 0{\cdot}021$, Fig. 2). Across the 20 non-exposed colonies, a total of 322 gynes and 7223 males were produced, corresponding to mean numbers (SE) of 16·1 (±6·7) and 361·2 (±61·6) per colony, respectively. In contrast, across the 20 neonicotinoid-exposed colonies, only 74 gynes and 4139 males were produced, corresponding to mean numbers (SE) of 3·7 (±2·0) and 207·0 (±33·3) per colony, respectively. Overall, these results indicate a population-level loss upon chronic neonicotinoid exposure of about 43% in males and 77% in queens. Male production was significantly decreased under neonicotinoid exposure ($F_{1,38} = 5{\cdot}29$, $P = 0{\cdot}027$), but was not affected by *Crithidia* exposure ($F_{1,37} = 0{\cdot}12$, $P = 0{\cdot}73$). Queen production is skewed in bumblebee colonies, resulting in many zeros. Coding queen production per colony as a binomial variable shows that fewer neonicotinoid-exposed colonies produced queens; however, this was not significant ($F_{1,38} = 3{\cdot}38$, $P = 0{\cdot}074$).

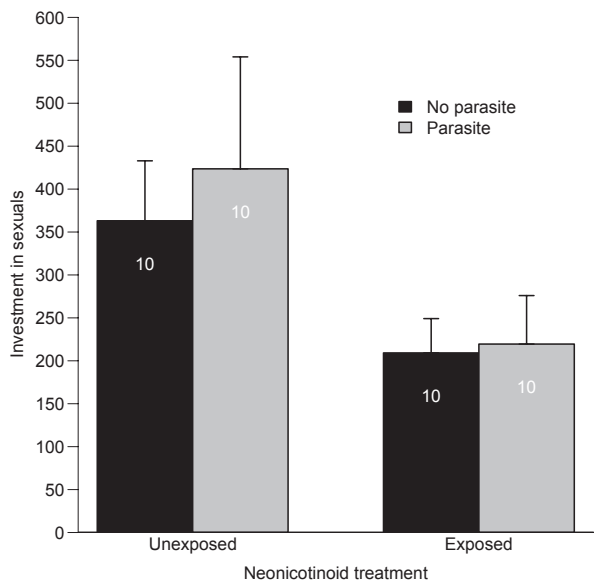

Fig. 2. Colony investment into sexual offspring in relation to corresponding neonicotinoid (thiamethoxam and clothianidin) and parasite (*Crithidia bombi*) treatments. To account for the greater cost of producing gynes, colony reproductive investment was calculated as the number of male offspring plus two times the number of gynes. Bars represent treatment means (+SE). Numbers inside the bars represent the number of colonies within each treatment group.

MOTHER QUEEN LONGEVITY

There was a significant interaction between the neonicotinoid and parasite treatments on mother queen longevity (Cox proportional hazard: $\chi^2_1 = 4{\cdot}76$, $P = 0{\cdot}029$). Mother queens of colonies that were exposed to both neonicotinoids and *C. bombi* had the lowest survival and a significantly decreased survival relative to controls [Fig. 3; hazard ratio = 4·82 (lower 95% CI = 1·15; upper 95% CI = 20·25)].

POLLEN AND SUGAR WATER COLLECTION

Sugar water collection per bee per week was influenced by week of the experiment ($F_{8,295} = 10{\cdot}28$, $P < 0{\cdot}001$) and by neonicotinoid exposure ($F_{1,34} = 20{\cdot}79$, $P < 0{\cdot}001$). Sugar water collection was consistently lower across all weeks in neonicotinoid-exposed colonies (Fig. 4). There was a significant interaction between neonicotinoid exposure and week of the experiment for the amount of pollen collected per bee per week ($F_{8,295} = 4{\cdot}66$, $P < 0{\cdot}001$). Pollen collection was initially the same in unexposed and exposed colonies, but diverged over the course of the experiment, with exposed colonies collecting less pollen per week per bee (Fig. 5).

PARASITE INFECTIONS

All checked bees in C and N treatments were free of *Crithidia*, while 99·4% of inspected bees sampled from P and PN treatments ($n = 173$) showed established infections, indicating that the parasite exposure treatment was effective in infecting the colonies. There was no significant effect of neonicotinoid treatment ($F_{1,168} = 1{\cdot}58$, $P = 0{\cdot}21$)

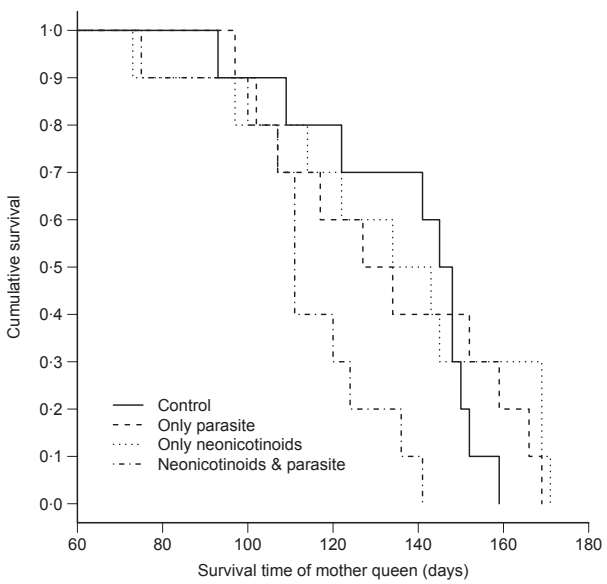

Fig. 3. Cumulative survivorship of mother queens across the different treatment groups ($n = 10$ for each line). The *x*-axis is truncated as all mother queens survived the first 60-day period following the initiation of the experiment.

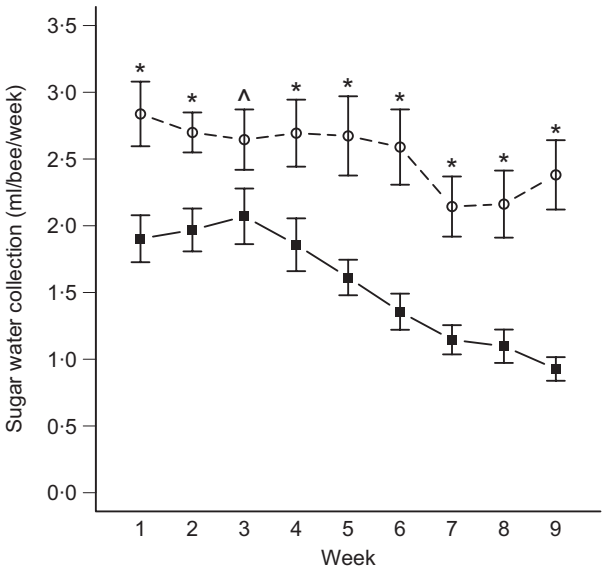

Fig. 4. Chronic dietary neonicotinoid (thiamethoxam and clothianidin) exposure and sugar water collection (millilitre per worker bee per week) across the 9 weeks of worker production after initiation of the experiment. Dashed lines and open symbols represent non-exposed colonies (n = 20), while solid lines and filled symbols represent exposed colonies (n = 20). Points show mean (±SE) sugar water collection. Symbols above bars represent the results of individual *post hoc* t-tests for each time point (*$P < 0{\cdot}05$, ^$0{\cdot}05 < P < 0{\cdot}1$).

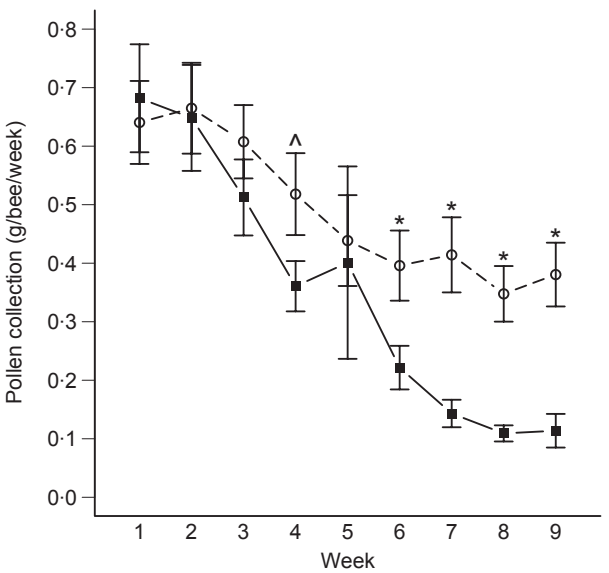

Fig. 5. Chronic dietary neonicotinoid (thiamethoxam and clothianidin) exposure and pollen collection (grams per worker bee per week) across the 9 weeks of worker production after initiation of the experiment. Dashed lines and open symbols represent non-exposed colonies (n = 20), while solid lines and filled symbols represent exposed colonies (n = 20). Points show mean (±SE) pollen collection. Symbols above bars represent the results of individual *post hoc* t-tests for each time point (*$P < 0{\cdot}05$; ^$0{\cdot}05 < P < 0{\cdot}1$).

or interaction between neonicotinoid treatment and time ($F_{1,168} = 2{\cdot}19$, $P = 0{\cdot}14$) on the number of parasite cells in the faeces.

Discussion

There is increasing concern about the impact of dietary neonicotinoid exposure on pollinators (Desneux, Decourtye & Delpuech 2007; Blacquière *et al*. 2012; Cresswell, Desneux & vanEngelsdorp 2012). Pesticides may interact with other important environmental stressors, for example parasites, to exacerbate negative effects that are imposed by the two factors in isolation. We investigated how the neonicotinoids thiamethoxam and clothianidin influenced crucial colony level traits in the bumblebee *B. terrestris*, and whether there are elevated detrimental effects through interactions with the prevalent trypanosome parasite *C. bombi*. Coinciding with recent reports focusing on imidacloprid (Gill, Ramos-Rodriguez & Raine 2012; Laycock *et al*. 2012; Whitehorn *et al*. 2012) and clothianidin (Larson, Redmond & Potter 2013), a detrimental impact on bumblebee colony growth and fitness was imposed by chronic dietary exposure to thiamethoxam and clothianidin. It truncated colony worker production (Fig. 1), decreased worker longevity and reduced reproductive investment (Fig. 2). While in most cases neonicotinoid treatment effects dominated, we demonstrate that combined exposure to both neonicotinoids and parasites can intensify effects on essential colony development traits, as seen in reduced mother queen survival (Fig. 3). Furthermore, our laboratory set-up suggested some anti-feeding effects, as inferred by lower collection of neonicotinoid-spiked sugar water from the outset (Fig. 4). The pattern of pollen collection (Fig. 5) on the other hand does not suggest anti-feeding effects for this resource, as collection levels only diverge later on in the experiment, and is more in line with a reduced effort in offspring rearing induced by the neonicotinoid treatment.

Bumblebees frequently exploit mass flowering crops such as oilseed rape and sunflowers as food resources (Westphal, Steffan-Dewenter & Tscharntke 2003; Goulson, Lye & Darvill 2008; Whitehorn *et al*. 2012). As systemic neonicotinoids are broadly used on these and other crops (Elbert *et al*. 2008; Jeschke *et al*. 2011), there is a conspicuous risk of bees being exposed to dietary trace residues. Although bumblebees are likely to also forage on non-crop alternatives, generalist bees tend to focus on high-reward and mass flowering crops (Westphal, Steffan-Dewenter & Tscharntke 2003; Holzschuh *et al*. 2011) successively available throughout the season in agricultural landscapes. Further, additional neonicotinoid exposure routes via non-crop resources may be possible (Krupke *et al*. 2012). Therefore, neonicotinoid exposure over most of the bumblebee colony cycle, as simulated here, while seemingly a worst-case scenario, could be realised in certain agroecosystems. Given the high prevalence of *C. bombi* (Shykoff & Schmid-Hempel 1991; Schmid-Hempel 2001; Gillespie 2010), combined pesticide and parasite pressure can be considered inevitable in the field. Our laboratory study gives insights into potential individual and interactive effects, while in field studies it would

be difficult to control the independence of both factors and rule out natural parasite infections (Imhoof & Schmid-Hempel 1999).

The accumulating evidence is that sublethal effects of neonicotinoid exposure on bees are expressed most strongly when performing challenging tasks such as foraging (Mommaerts *et al.* 2010; Belzunces, Tchamitchan & Brunet 2012; Gill, Ramos-Rodriguez & Raine 2012; Henry *et al.* 2012). Our experimental environment was rather benign with regard to such challenging tasks. Indeed, we found *C. bombi* infections alone (P treatment) did not result in measurable effects, which have otherwise been shown under stressful conditions (Shykoff & Schmid-Hempel 1991; Brown, Loosli & Schmid-Hempel 2000; Schmid-Hempel 2001; Brown, Schmid-Hempel & Schmid-Hempel 2003; Gegear, Otterstatter & Thomson 2005, 2006). Although our laboratory approach was different from the recent semi-field studies of Gill, Ramos-Rodriguez & Raine (2012) and Whitehorn *et al.* (2012) using imidacloprid, or the one of Larson, Redmond & Potter (2013) using clothianidin, we arrive at very similar findings of neonicotinoid-exposed colonies (N and PN treatments) exhibiting decreased reproductive output (Fig. 2) compared to non-exposed colonies (C and P treatments). Our findings both reinforce the generality of such effects for neonicotinoids outside of imidacloprid and add an interesting component to complement the recently provided mechanistic interpretation (Gill, Ramos-Rodriguez & Raine 2012). It was argued that impaired foraging efficiency in bumblebees, eventually reinforced by higher forager losses (Gill, Ramos-Rodriguez & Raine 2012), could primarily be responsible for fitness losses (Whitehorn *et al.* 2012). Indeed, there are clear relationships between resource allocation and colony growth, and between colony size and the initiation of sexual investment (Müller & Schmid-Hempel 1992; Imhoof & Schmid-Hempel 1999; Schmid-Hempel 2001; Gill, Ramos-Rodriguez & Raine 2012; Whitehorn *et al.* 2012). Interestingly, concurring with Gill, Ramos-Rodriguez & Raine (2012), truncation of worker production emerged after 3 weeks of chronic neonicotinoid exposure (Fig. 1), coinciding with the time needed for a whole brood cycle. However, considering that our laboratory approach provided a low-complexity environment with *ad libitum* food, our results suggest additional adverse effects on colony development. It remains unclear whether neonicotinoids cause disruptions in brood development directly, or whether workers have reduced brood care abilities. Increased numbers of emptied brood cells and dead larvae documented in the outside boxes of neonicotinoid-exposed colonies (A. Fauser-Misslin, personal observation) could suggest increased susceptibility to bumblebee brood. However, higher brood mortality could be similarly expected if adult workers were neglecting the brood, for example, as a consequence of increased lethargy. Moreover, thiamethoxam and clothianidin concentrations expected to represent sublethal oral dosages (Mommaerts *et al.* 2010; Blacquière *et al.* 2012) resulted in significantly decreased bumblebee worker longevity within colonies upon chronic exposure, as previously demonstrated (Tasei, Lerin & Ripault 2000).

Interestingly, while there is no evidence that foraging bumblebees discriminate between thiamethoxam-treated and untreated oilseed rape in the field [The Food & Environment Research Agency (Fera) 2013], the laboratory study of Elston, Thompson & Walters (2013) has found that the collection of sugar water spiked with thiamethoxam depends on the concentration. The here-observed differential sugar water collections from the outset of the experiment (Fig. 4) do not rule out the mechanistic involvement of anti-feeding effects. It is plausible that decreased provisioning could at least partly explain the results seen on the colony level, as the bees did not have access to an alternative non-contaminated foraging source. Reduced collection of neonicotinoid-spiked sugar water could result from: (i) avoidance by the foraging workers, (ii) collecting readily but then choosing not to feed it to the brood (resulting in more stores and subsequently triggering less foraging) or (iii) worker lethargy and brood negligence with an immediate drop in further collection. These would have different implications and need more detailed exploration. The initial equal collection of pollen across treatment groups (Fig. 5) suggests that anti-feeding effects do not apply to all resources. The subsequent decrease in pollen collection in neonicotinoid-exposed colonies can probably be attributed to reduced offspring rearing investment.

Mounting evidence of global pollinator declines in general (Potts *et al.* 2010) and bumblebees in particular (Goulson, Lye & Darvill 2008; Grixti *et al.* 2009; Cameron *et al.* 2011) is indicative of increasingly less viable populations (Carvalheiro *et al.* 2013). In addition to wider biodiversity risks, the prospect of continuous wild pollinator population declines is of economic and food security concern (Garibaldi *et al.* 2013). Bumblebees are increasingly used for pollination management (Goulson, Lye & Darvill 2008) and offer a way of reducing pollination deficits associated with ongoing honeybee losses (Winfree *et al.* 2007; Aizen *et al.* 2008; Potts *et al.* 2010). This study adds to the accumulating experimental evidence on the link between chronic exposure to neonicotinoids and reduced bumblebee fitness (Gill, Ramos-Rodriguez & Raine 2012; Laycock *et al.* 2012; Whitehorn *et al.* 2012; Larson, Redmond & Potter 2013), which could be relevant in field environments, including long-term population impacts. Moreover, as similarly indicated in honeybees (Alaux *et al.* 2010; Vidau *et al.* 2011), we demonstrate the plausibility of increased detrimental effects from combined neonicotinoid and parasite exposure. Given that bumblebee worker foraging performance and survival, as well as colony fitness, are adversely affected by both neonicotinoids and *C. bombi*, the latter at least conditionally (Brown, Loosli & Schmid-Hempel 2000; Schmid-Hempel 2001; Brown, Schmid-Hempel & Schmid-Hempel 2003; Gegear, Otterstatter & Thomson 2005, 2006), impacts may be

compounded by infections with this parasite. Although our experiment revealed no significant interactions between the combined pressures of these neonicotinoids and *C. bombi* on colony growth relative to the overall strong neonicotinoid effects alone, mean worker longevities and the likelihood of daughter queen production were lowest in the PN group compared to all other treatments. Due to the condition dependence of *C. bombi*, enhanced detrimental fitness effects of combined exposure are likely to be more strongly pronounced under less benign natural conditions. In this context, our finding of a negative interactive effect of neonicotinoids and *C. bombi* on mother queen longevity (Fig. 3) represents evidence for an elevated detrimental impact on crucial bumblebee colony traits, an aspect that clearly deserves further research. Queen survival until and throughout the reproductive phase is pivotal because colony fitness is positively correlated with the length of the reproductive period (Imhoof & Schmid-Hempel 1999), and premature mother queen death inevitably suppresses daughter queen production. Our experimental colonies were first exposed to neonicotinoids and parasites at the 10-worker stage. However, the greatest impact by *C. bombi* is on colony founding by queens (Brown, Schmid-Hempel & Schmid-Hempel 2003), an aspect not investigated here. Considerable proportions of spring queens can be infected with *C. bombi*, and there is a realistic risk of exposure to neonicotinoids during this critical early season phase that could exacerbate already high rates of *C. bombi*-imposed colony founding failure. Further, an earlier exposure to these interacting detrimental pressures could result in mother queen death prior to the initiation of sexual offspring production.

In conclusion, although presently mandatory guidelines for pesticide risk assessment and testing side effects of plant protection products on pollinators based on the tiered approach are comprehensive (OECD 1998a,b; OEPP/EPPO 2010a,b), a number of recent studies demonstrate that there may be important limitations. Partly referring to the recently published guidance document of the European Food Safety Authority (http://www.efsa.europa.eu/en/efsajournal/doc/3295.pdf), three major aspects, which previously remained insufficiently implemented or untargeted at all, thus deserve more detailed future consideration:

1. sublethal and chronic effects under more complex/realistic laboratory and (semi-)field conditions, selectively including direct assessments of specifically meaningful parameters, for example detailed estimates of homing and foraging efficiency, and vital endpoints such as reproductive investment.
2. reliable laboratory or semi-field estimates of interactive effects between pesticides and common parasites under chronic exposure.
3. expanding mandatory, exclusively honeybee-based test systems for pollinators with a broader diversity of key non-*Apis* bees, such as selected representatives of bumblebees and solitary bees.

Some non-*Apis* bees, exhibiting relatively simple life cycles, appear to be suited for assessing reproductive investment under higher tier testing. This response is covered in some other non-target arthropods, but virtually not quantifiable in honeybees. Fitness can be considered as a sensitive and meaningful endpoint in evaluating effects of pesticides on pollinators. Sexual reproductive output is specifically informative to infer population-level consequences when causal mechanistic manifestations of sublethal effects remain cryptic. Yet, its assessment critically requires complementing present standard testing periods of chronic exposure with monitoring of whole life/colony cycles. A consensus on principal future directions for revised pesticide risk assessments guidelines across stakeholder panels is needed. Implementing the manifold benefits of selected non-*Apis* bees, for example building on procedures such as the bumblebee colony fitness assay here, that could be also modified as semi-field (tunnel) approach, and in any case be combined with subsequent field monitoring (i.e. laboratory/semi-field to field approach with restricted chronic exposure periods), appears to be essential in order to safeguard pollinator populations that provide vital ecosystem services.

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