Traces of a neonicotinoid pesticide stimulates activity in honey bee colonies, but not productivity

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Article

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Abstract

How neonicotinoid contamination effects honey bees remains controversial. Studies have yielded contradictory results, and few have examined effects on colony development. Here we report the results of a comprehensive five-year study of the effects of the neonicotinoid imidacloprid on honey bee colonies. Colonies fed 5 ng/g (ppb) imidacloprid showed increased brood production, lower temperature variability, higher CO2 production and had more foragers compared to control colonies. Even so, treatment did not affect adult bee numbers or average hive temperatures, and did not increase food stores, daily food acquisition or colony survivorship. Imidacloprid contamination increased colony metabolism without improving colony productivity, which is consistent with the pharmacological action of imidacloprid as a stimulant. Our findings explain why some studies have reported no, or even positive, effects of neonicotinoids. We provide an explanation for the diverse effects of pesticide contamination on honey bees, and a thorough understanding of how colonies are impacted.

Introduction

The extent to which insecticides harm non-target beneficial insects continues to be highly controversial[1-5]. Some countries have banned neonicotinoid insecticides due to concerns about the negative impacts of neonicotinoid residues on pollinators, particularly honey bees[6]. These bans have in turn caused some workers to raise concerns about the damaging effects of insecticides that have been substituted for the neonicotinoids[3,7]. Controversies remain because it is still unclear how honey bees, and bee colonies, are affected by the low concentrations of neonicotinoids to which they can be exposed when foraging on crops[1,8].

Imidacloprid is the most commonly used insecticide in the world, and from a toxicological perspective it is one that poses an important risk for honey bees[9,10]. Honey bees are exposed to imidacloprid through ingestion of contaminated nectar, pollen, stored honey and water, and possibly aerial dusts. Harmful but sublethal effects of imidacloprid have been described from concentrations as low as 0.1 ppb[11]. Imidacloprid is frequently found within honey bee hives[12], and chronic imidacloprid exposure levels have been reported to be as high as 10 ppb, although field concentrations are highly variable[12].

Studies of the effects of field-relevant concentrations of neonicotinoids on honey bee colony performance and survival have reported inconsistent or contradictory results. Colonies exposed to clothianidin had more eggs in Germany, fewer eggs in Hungary, and fewer workers and storage cells in the UK[1]. Honey bee colony mortality was positively correlated with imidacloprid usage in England and Wales[13]. Colony weight gain was reduced in colonies exposed to neonicotinoid treated crops in the UK[14]. In North America imidacloprid has been shown to negatively affect brood production, queen replacement, foraging activity, winter survivorship, social immunity, and increase Varroa densities, when applied at sublethal concentrations[8,15], and to positively affect flight activity and colony thermoregulation[16]. In Australia honey bees exposed to 5 ppb imidacloprid were found to forage at a younger age but to

accumulate less food on the long term[17,18]. Additional effects that have been reported include: increased water consumption[11], decreased foraging performance[19], higher bee cluster temperatures[20] and reduced learning performance when mixed with another pesticide[21]. Even so, other studies have reported no individual effects at these low levels[19,22,23]. These contrasting results point towards either increased false-discovery rates due to the low sample sizes of these studies[3], or towards complex and dose-dependent interactions between the environment, honey bees, and neonicotinoid insecticides.

As colonial insects, honey bees do not typically experience stressors as individuals[24]. Their social environment can be a powerful buffer of stress[25]. Documenting behavioral impacts on individuals may not translate to consequences that *matter* for agriculture and apiculture, such as colony behavior and survivorship. For growers the issues of concern are whether neonicotinoids affect crop production by affecting pollinators and pollination and whether neonicotinoids are effective in controlling pest outbreaks. For beekeepers the issues of concern are whether neonicotinoids deplete their colonies of bees or reduce colony productivity.

To better understand how imidacloprid affects honey bee colonies we conducted five field experiments, involving a total of 54 bee colonies, measuring the effects of chronically feeding syrup adulterated with 5ppb imidacloprid on bee colony development and behavior. In each experiment, one group of colonies were fed a six-week regimen of sugar syrup adulterated with imidacloprid at 5 ppb, and another group were fed the same regimen with blank sugar syrup. Treatments were intended to simulate nectar collection by the colonies. No commercial agriculture existed within 10 km of the sites. We collected data from hive assessments, including numbers of adult bees and capped brood cells and food reserves; and from sensors, including continuous hive weight, internal temperature and internal CO_2 concentration. We show that at this low level imidacloprid stimulated bee activity, which may explain some of the positive effects of the pesticide that have been reported. Even so, the increased activity of the colonies did not translate to increased colony productivity.

Results

A summary of statistical results is provided (Table 1). Colonies in both groups consumed on average at least 92% of the treatment syrup in all years except 2017, when control colonies consumed 80±9% and imidacloprid colonies consumed 86±8% of the syrup. The number of capped brood cells (a measure of reproduction) was significantly affected by both treatment and year (Figure 1). The imidacloprid group produced more brood. Colonies exposed to imidacloprid had on average 6326±504 capped cells for the first two post-treatment sampling occasions, while control colonies had on average 5400±504 capped cells. Despite the higher brood production in the imidacloprid group, there was no difference in adult bee mass between groups. The change in the amount of food stores and Varroa mite fall were likewise not affected by treatment but significantly differed among experiments (Table 1). This was likely due to weather and forage availability. NEB dry weights were not affected by treatment or year. Raw data on colony survivorship did not show large differences between groups (SI Figure 1).

Table 1. Summary of statistical results. P-values for treatment, year and their interactions in analyses of variance. Data transformed as log values where necessary. In the Treatment and Year columns, "+" means colonies given imidacloprid had higher values and "-" lower values. "Wt." means weight; "temp." means temperature; "avg." means average; "ampl." means within-day amplitudes. Column "Covariate" indicates the pre-treatment measurements used as covariates. Significant P values are in bold. Full results are provided (SI Tables 2-19)

Variable	Time periods	Treatment	Year	Covariate
No. adults	Sept., Oct.	P=0.3534	**P=0.0004**	No. adults
No. brood cells	Sept., Oct.	**P=0.0256 (+)**	**P<0.0001**	No. brood cells
Food wt. change-Fall (g)	Sept. to Oct.	P=0.6804	**P=0.0001**	Food wt.
Food wt. change-Winter (g)	Oct. to Feb.	P=0.9294	**P<0.0001**	Food wt.
NEB dry wt. 2016-18 (g)	Aug., Sept.	P=0.9606	P=0.2669	NEB dry wt.
Log daily Varroa fall	Post treatment	P=0.4160	**P<0.0001**	Mite fall
Log temp. avg. Fall (°C)	1 Nov.–31 Dec.	P=0.9453	P=0.1328	Temp. avg.
Log temp. ampl. Fall (°C)	1 Nov.–31 Dec.	P=0.9335	P=0.0716	Temp. ampl.
Log temp. avg. Winter (°C)	1 Jan.–8 Feb.	P=0.2363	P=0.3822	Temp. avg.
Log temp. ampl. Winter (°C)	1 Jan.–8 Feb.	**P=0.0338 (-)**	P=0.3804	Temp. ampl.
Log avg. CO_2 2018-19 (ppm)	1 Sept.-30 Nov.	**P=0.0287 (+)**	**P=0.0055**	CO_2 avg.
Log CO_2 ampl. 2018-19 (ppm)	1 Sept.-30 Nov.	P=0.3212	**P=0.0074**	CO_2 ampl.
Daily wt. change-Fall (g)	1 Sept.–31 Oct.	P=0.2034	**P<0.0001**	Adult wt.
Log avg. CO_2 2018-19 (ppm)	July – Nov.	**P=0.0287 (+)**	P=0.4333	Adult wt. *
Daily wt. change-Winter (g)	1 Dec.-31 Jan.	P=0.9539	**P<0.0001**	Adult wt.
Forager departure wt. loss (g)	1 Sept.–31 Oct.	**P=0.0300 (+)**	**P<0.0001**	Adult wt.
Dawn break point (h)	1 Sept.–31 Oct.	P=0.6598	**P<0.0001**	Adult wt.
Dusk break point (h)	1 Sept.–31 Oct.	P=0.1468	**P<0.0001**	Adult wt.

* Adult weight values measured at the same time as the CO_2 values, i.e. at hive assessments, were used as covariates, rather than pre-treatment values.

Temperature amplitudes were lower in the imidacloprid group, suggesting more controlled thermoregulation in that group. Average daily temperatures were not different (Figure 2, Table 1). We performed two analyses of daily CO_2 concentrations. In the first, average daily values were analyzed from

Sept. to Nov., with pre-treatment values as covariates. In the second only concentrations at the time of hive evaluations were considered, with associated adult bee masses as covariates. In both analyses overall log CO_2 concentrations were higher in the imidacloprid group than the control group (Figure 3, Table 1): in the first analysis 5238±187 ppm compared to 4399±170 ppm; and in the second analysis 4382±423 ppm compared to 3461±344 ppm. Hive CO_2 concentrations were also inversely proportional to adult bee mass (imidacloprid: slope=-0.151±0.027, adjusted r^2=0.45; control: slope=-0.104±0.026, adjusted r^2=0.27) (Figure 4).

Within-day measures of hive weight changes were analyzed using piecewise regression (Table 1). We have shown previously that the transient rapid decline in mass at dawn is due to the departure of forager bees from the hive[26] and this parameter was affected by treatment. Imidacloprid treated hives lost more weight during forager departure (118±12 g) than control hives (100±9 g) (Figure 3, Table 1). These values were calculated from 1 Sept. to 31 Oct. and so include many days when foraging was poor (nectar flows in general ceased by early Oct.). Some days each year were excluded from the analysis because of hive manipulations, rain, or poor curve fits[27]. These results indicate that imidacloprid colonies overall had a larger population of active foragers despite not having larger bee populations.

Imidacloprid was stable in honey stores for months after the treatment (SI Table 1). The high concentration of imidacloprid in 2017 likely resulted from imidacloprid concentration by bees due to low nectar yield. In 2017 the October food stores in colonies in the imidacloprid group had on average only 7.18±1.00 kg, whereas in 2014, 2016, 2018 and 2019 colonies in that group had 14.96±1.00, 13.30±1.20, 17.16±1.67 and 17.82±1.01 kg, respectively.

Discussion

Here we report a comprehensive and long-term study of colony-level effects of a chronic, low level neonicotinoid exposure on honey bee colonies. We believe we now understand why studies of colony-level effects of low neonicotinoid doses have yielded variable and contradictory results. The best explanation for the effects we found is that imidacloprid increases adult bee activity, but in our experiments this did not translate to better colony outcomes.

While there is still debate about the level at which bee colonies are exposed to imidacloprid in the field, we focused on exposure to 5 ppb imidacloprid, which is at the lower range of bee exposure estimates[12]. Colonies provided with this imidacloprid treatment had significantly higher brood production in the two months after treatment, producing about 17% more brood on average than control colonies. Even so this did not translate to higher adult bee populations, suggesting an increased mortality of brood, adults or both. Imidacloprid treated colonies also had a larger proportion of active foragers, as measured by the dynamic change in weight at dawn as foragers leave the hive. Even though the foraging force was larger in the treated than in control colonies, it did not translate into increased food gain.

We observed lower temperature variability in the imidacloprid treated colonies. Honey bees actively heat or cool the hive to maintain an optimal temperature for immature bees[28,29]. Tighter regulation of the brood nest temperature indicates a greater investment by adult bees in thermoregulation. The differences seen were greatest midwinter, typically when bees invest energy to heat the colony.

Daily hive CO_2 concentrations were inversely proportional to the adult bee population sizes, i.e. larger colonies had lower hive CO_2 concentrations than smaller colonies. Clearly larger colonies would produce more CO_2, but having more workers could also ventilate better. While imidacloprid treatment did not affect adult bee numbers, hive CO_2 concentrations in small colonies in the imidacloprid group were measurably higher than those of small colonies in the control group, yet among larger colonies the two groups were indistinguishable. This suggests that the imidacloprid treatment increased CO_2 production but did not affect CO_2 tolerance.

All of these effects indicate increased activity in the imidacloprid treated colonies. This interpretation is consistent with the molecular action of imidacloprid as a competitive agonist of nicotinic acetylcholine receptors in the insect nervous system[30-32]. At very low levels imidacloprid excites neural activity and increases neural oxidative stress[33], and such exposure has been associated with stimulant effects[34,35]. The stimulant effects of imidacloprid would explain the changes in colony physiology seen here. The pharmacological action of imidacloprid is not dissimilar to nicotine[37], which increases general arousal, motor responsiveness and sensitivity in both insects and mammals at low doses. This would explain both the increased investment in thermoregulation and increased CO_2 levels in treated colonies.

These neuroexcitatory effects would also explain behavioral changes observed here. Stimulation of muscarinic acetylcholine receptors by pilocarpine induces a precocious onset of foraging in honey bees[38]. Precocious foraging is a common stress response in bees and triggered by disease and starvation[39-41]. The 5 ppb imidacloprid treatment we used here has also been found to induce precocious foraging[18]. In this study we did not measure the age of foraging onset, but we did observe increased foraging activity. From this pharmacological perspective we would expect the effect of imidacloprid on bees to be highly dose dependent. Indeed, at higher levels it overexcites central and peripheral neural circuits and leads to motor dysfunction and death[36]. At the colony level, treatment with a higher dose of imidacloprid at 100 ppb in syrup has been shown to reduce adult bee and brood populations, and increase temperature variability[16].

The effects documented here are consistent with hormesis, a biphasic physiological response to increasing concentrations of a compound[42-44], which is usually excitatory at low doses and inhibitory at higher doses. Such effects may imbalance the fragile metabolic optimum that bee colonies depend on to maintain homeostasis and growth. We emphasize that the increased foraging or temperature regulation triggered by imidacloprid in this study did not significantly improve outcomes for colonies. It is possible that, in some environments, spending more energy foraging and hive thermoregulation could benefit a colony while in other environments it may not. Our findings offer a simple way to explain why previous

studies have found highly variable and dose dependent effects of sublethal neonicotinoid exposure. We offer a single physiological framework to understand the diverse and dose-dependent effects on honey bees of neonicotinoid insecticides at sublethal concentrations.

Materials And Methods

Sucrose syrup preparation. To produce syrup, sucrose was added to distilled water in a 20 L bucket and mixed until sugar was dissolved to produce a solution of 1:1 sucrose weight to water volume. To produce syrup with imidacloprid, 1 kg sugar was dissolved in 900 mL distilled water to produce 1.9 kg “short” solution. A spike solution was prepared by first dissolving 1.0 mg imidacloprid (Pestanal, CAS # 138261-41-3) in 100 mL distilled water, then mixing 1 mL of that solution into 99 mL distilled water to achieve a 100 mL spike solution. The spike was then added to 1.9 kg short sucrose solution to achieve 2 kg of 5 ppb imidacloprid syrup.

Field experiments. The 2014 experiment[16] and 2016 experiment[17] have been described previously and were conducted at apiaries in the Santa Rita Experimental Range (31°46'39"N, 110°51'46"W). The 2017, 2018 and 2019 experiments were conducted <1 km north of the 2014 site; data for the control treatment in the 2017 and 2018 studies have also been described previously[45]. The apiaries were surrounded by native mesquites *(Prosopis spp.*), cacti (e.g., *Opuntia spp.*), creosote (*Larrea tridentata*) and other wildflowers.

Honey bee colonies were purchased as 1kg bee packages (C.F. Koehnen & Sons, Inc., Glenn, CA 95943) for the first three experiments, and as colonies (Marquette Apiaries, Winchester, CA 92596) for the last two. Colonies were kept in painted, 10-frame, wooden Langstroth boxes (43.7 l capacity) with migratory wooden lids. Each package was given 4 frames of capped honey, 2 frames empty drawn comb, 3 frames of foundation and a frame feeder. Colonies established as packages were all immediately fed 2 kg sugar syrup (1:1 w:w) and 250 g pollen patty, made at a ratio of 1:1:1 corbicular pollen (Great Lakes Bee Co.): granulated sugar: drivert sugar (Domino Foods). Hives were placed on electronic scales (Tekfa model B-2418 and Avery Weigh-Tronix model BSAO1824-200, max. capacity: 100 kg, precision: ±20g; operating temperature: -30ºC to 70ºC), powered by deep-cycle solar powered batteries, and linked to dataloggers (Hobo UX120-006M, Onset Computer Corporation, Bourne, MA, USA) with weight recorded every 5 minutes (every 15 min in 2014). Hives were organized in groups of 4-6 hives; groups were >3 m from each other and within groups hives were at least 0.5m apart.

In early July temperature sensors (iButton Thermochron, precision ±0.06°C) enclosed in plastic cassettes (Thermo Fisher Scientific, Waltham, MA) were stapled to the center of the top bar on the middle frame in the bottom box and set to record every 15 min. In the 2018 and 2019 experiments, CO_2 probes (Vaisala Inc., Helsinki, Finland), calibrated for 0-20% concentrations, were placed on top of the center frames in the top box of five hives in each treatment group and set to record every 5 min. At that time, paperboards coated with petroleum jelly and covered with mesh were inserted onto hive bottom boards and left 2-3 days to monitor *Varroa* mite fall[46].

Hives were assessed before assignment to treatment groups using a published protocol[26,47]. Briefly, each frame was gently shaken to dislodge bees, photographed (Rebel SL1, Canon USA, Inc., Melville, NY), weighed (EC15, OHaus), and replaced. During this first assessment, all hive components (i.e. lid, inner cover, boxes, bottom board, etc.) were also shaken free of bees and weighed. The total adult bee population weight was calculated by subtracting the combined weights of hive components free of bees from the total hive weight with bees recorded at midnight prior to the inspection. In addition, 3-5 g of honey were collected from each hive into 50 ml tubes and stored at -80ºC; samples collected in July before treatment were pooled and subjected residue analysis of pesticides and fungicides by the Laboratory Approval and Testing Division, USDA.

After the first assessment, hives were assigned to groups based on adult bee mass and location (to avoid clumping) and each group randomly assigned a treatment. Just prior to treatment all broodless frames with food stores were replaced with empty drawn comb. Colonies were then fed 2-3 kg treatment syrup twice per week for six weeks, from mid-July onwards. Syrup consumption per colony was recorded. Hives were assessed every 5-6 weeks after the treatment period until Nov., and once more in Feb. At each assessment, hive lid and inner cover weights were compared to previous values to correct wooden parts for moisture content and improve adult bee estimates.

We sampled NEBs before and after the treatment period by pressing an 8 cm x 8 cm wire cage into capped brood, collecting trapped NEBs into 50 mL centrifuge tubes 24 h later, and then storing them at -80°C. To determine dry weight, five bees per hive per date were placed in Eppendorf tubes and weighed before and after drying for 72 h at 60°C. Varroa mite fall was measured within a few weeks after treatment and colonies were treated in Oct. or Nov. using an amitraz-based product. Ambient weather data was obtained from AmeriFlux US-SRM Santa Rita Mesquite, https://doi.org/10.17190/AMF/1246104.

Data analysis

Mass values were converted to adult numbers by assuming an average of 0.11 g per adult. The area of sealed brood per frame was measured from photographs using ImageJ version 1.47 software (W. Rasband, National Institutes of Health, USA) or CombCount[48] and multiplied by 4.1 cells per cm^2 [47] to estimate capped cell number. Stored food mass was calculated thus:

1. Subtracting the weight of an empty drawn comb, about 555 g[47], from the weight of each frame (negative values counted as zero) to estimate brood + food weight;
2. Adding the brood + food frame weights for each hive;
3. Multiplying the brood surface area by 0.77 to estimate g brood mass[47] and subtracting that value from the brood + food weight.
4. Dividing the change in the stored food mass between the Oct. and Feb. hive assessments by the number of days between those assessments.

Adult bee, brood cell and food resources were subjected to repeated-measures MANOVA with treatment, year, sampling date, and all 2-way interactions as fixed effects and with pre-treatment values as covariates, where possible, to control for pre-existing differences (Proc Glimmix, SAS Inc. 2002). Post-hoc contrasts with Bonferroni correction were reported for significant main effects. Analyses of NEB dry weight and Varroa fall were limited to a single sampling occasion immediately after the end of treatment application. Temperature and CO_2 data were transformed into daily average and within-day detrended data, calculated as the difference between the 24 hour running average and the raw data. Sine curves were fit to 3-day subsamples of detrended data[47] and MANOVA analyses conducted on daily averages and sine amplitudes. To improve the detection of treatment effects across seasons, continuous data were divided into different time periods for analysis (see Table 1). Hive CO_2 concentrations were also analyzed using a second method: CO_2 concentrations at the time of hive evaluations were estimated by averaging data over three days prior to evaluations (July concentrations at start of study obtained three days post evaluation) and subjected to the MANOVA analysis described above with adult bee mass as covariates. Figures were made in R v4 using tidyr v1.1.0, dplyr v0.8.5, cowplot v1.0.0 and ggplot 2 3.3.1.

Continuous hive weight data per day were analyzed by piecewise regression using a bootstrapping procedure[49] after subtracting the hive weight at midnight from each subsequent weight value over the next 24 h[50]. Data were fit with piecewise regression, yielding estimates for 4 break points, 5 slope values and the adjusted r^2. Four parameters were used: 1) night slope S_N (rate of hive weight change due to moisture loss from midnight until dawn); 2) dawn break point t_D (start of daily foraging activity); 3) slope of the 1st segment after dawn S_M (rate of morning hive weight change); and 4) dusk break point (end of daily foraging activity). S_M was attributed to both forager departure and moisture loss (i.e. nectar drying, respiration). To estimate weight change due to forager departure, the effect of moisture weight change was removed thus:

$$\Delta F = S_M(t_{D+1} - t_D) - S_N(t_{D+1} - t_D) \qquad [1]$$

with ΔF the hive weight change due to forager departure, and t_{D+1} the time of the break point following the dawn break point t_D. ΔF, t_D, and the dusk break point were used as response variables in MANOVA analyses (see above). Days with rainfall >3 mm, slopes <-0.4kg/min, and forager weight change > 0 (indicating hive weight gain) were excluded. Analyses of hive weight data were limited to approximately 2 months after the end of treatment to focus on the active season.

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Declarations

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Author Contributions

WGM, MW, and JJA designed the experiments. WGM, TC and MW collected the data. WGM, TC, ABB analyzed the results. ABB, TC, WGM and JJA wrote the manuscript.

Figures

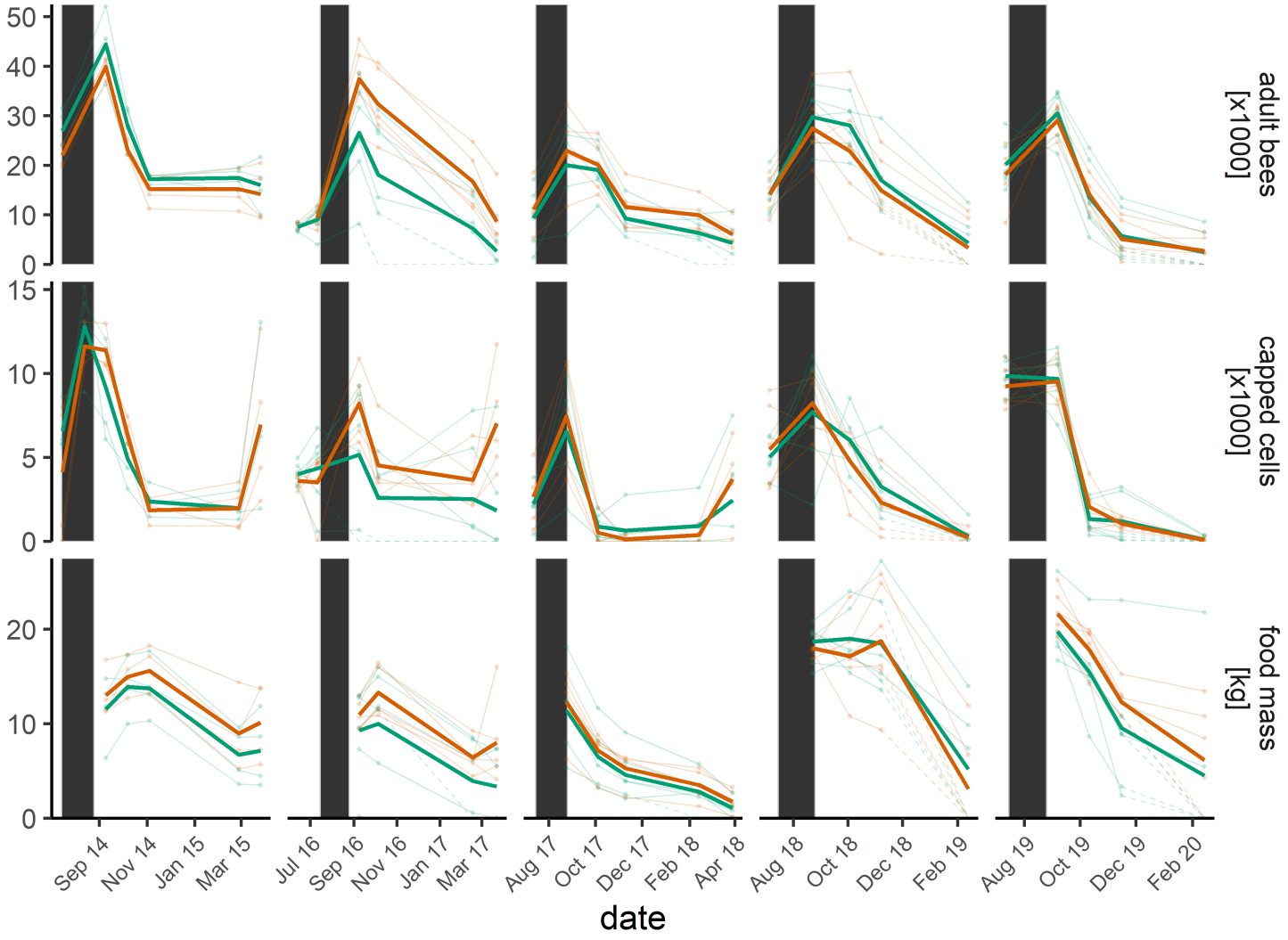

Figure 1

Imidacloprid treatment (red) had no significant effects on the adult bee population or food mass accumulated compared to the control (green), but increased brood amounts slightly in the first months following treatment (Sept. Oct.). Black columns show treatment period. Thick lines show group averages, thin transparent lines individual hive data, dashed lines last measurements before colony death.

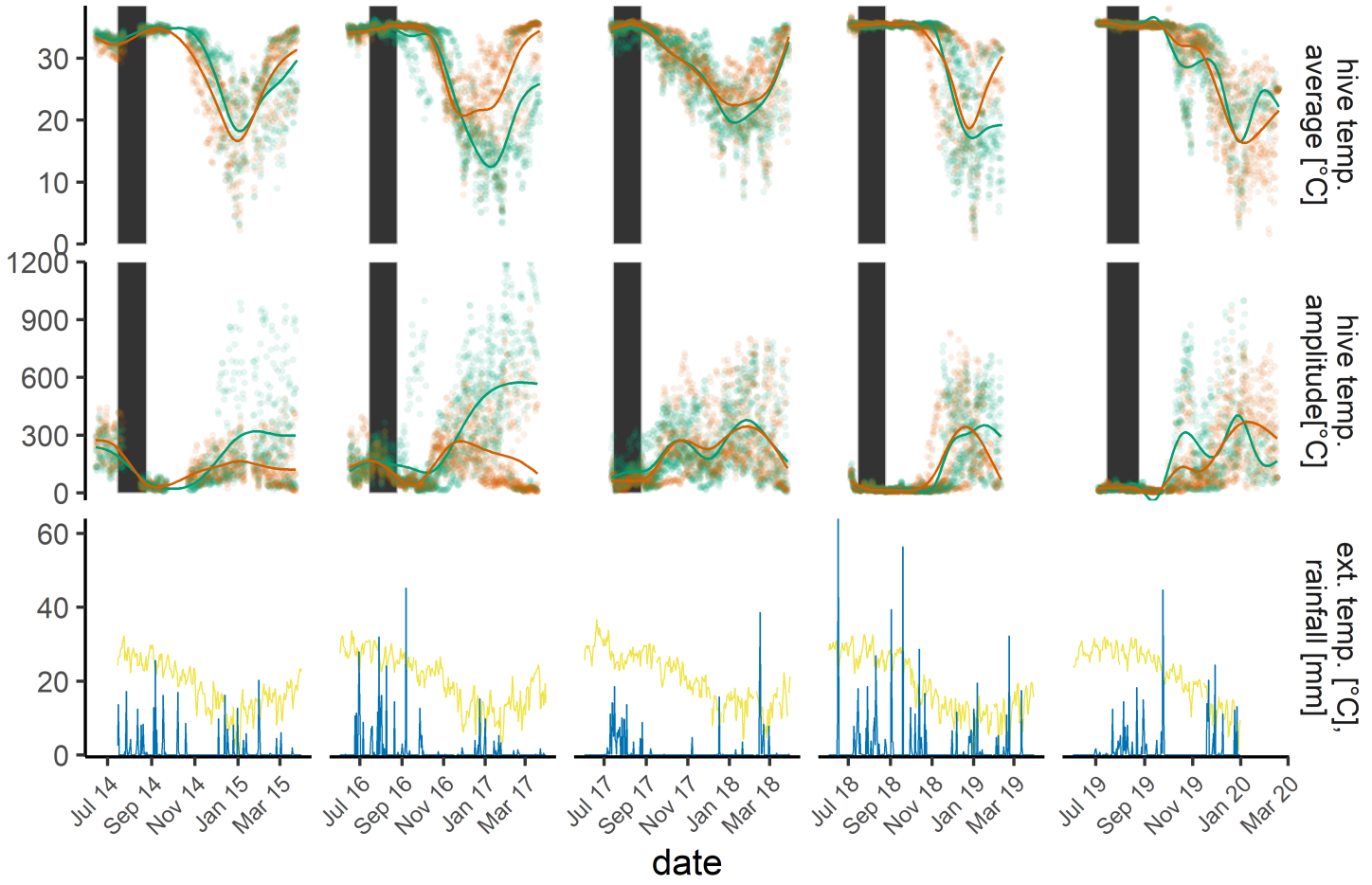

Figure 2

Average temperatures in bee colonies exposed to imidacloprid (red) were not significantly different from those of control colonies (green), but imidacloprid treated colonies were less variable indicating greater temperature control with variations of 2.8±0.1°C on average, compared to control colonies that varied by 4.0±0.2°C on average. External temperature (yellow) and total daily rainfall (blue) are given below in-hive measurements. Black columns show treatment period. Dots show raw data, smoothed lines default ggplot2/R GAMs.

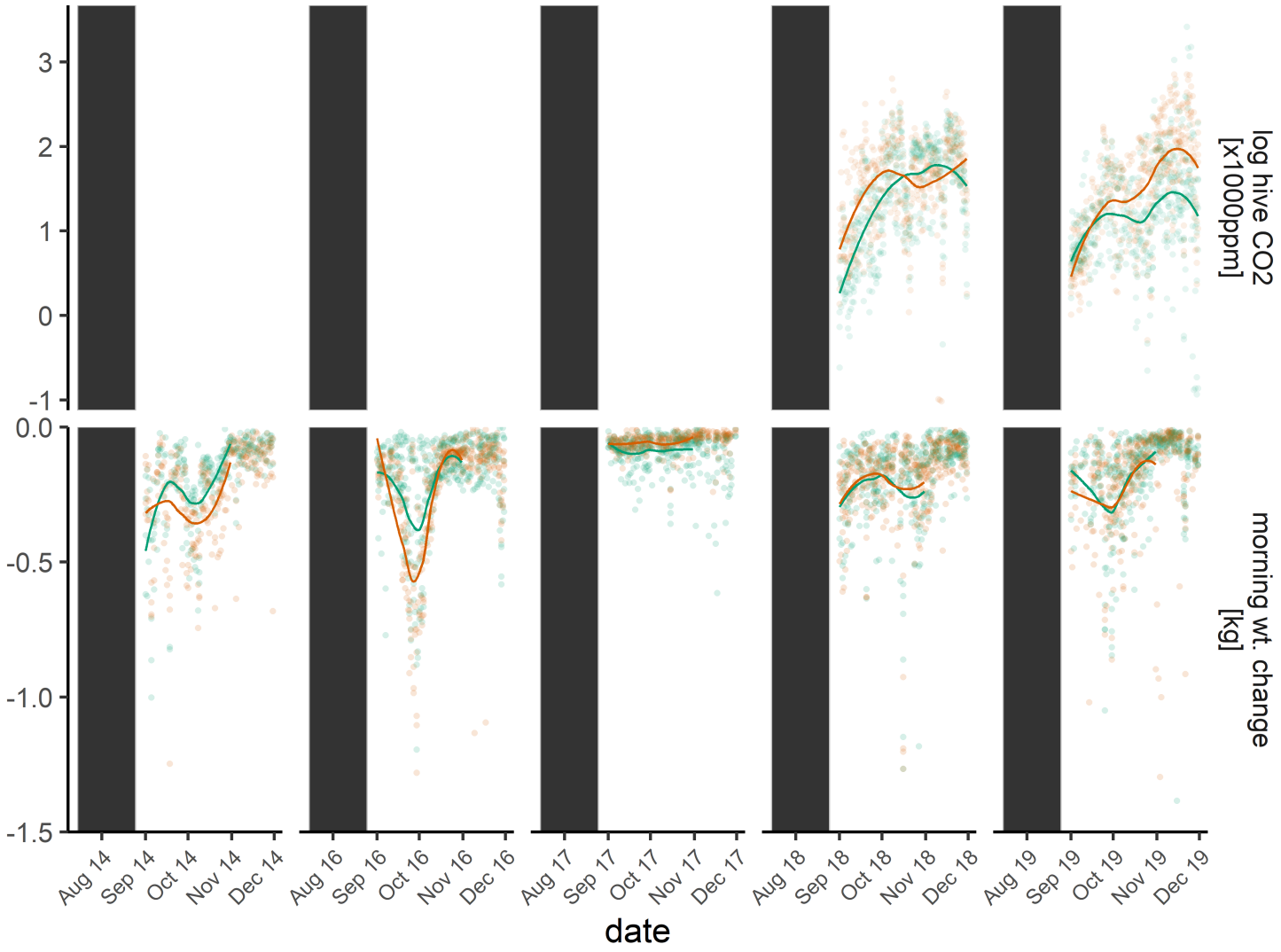

Figure 3

Upper graph: average CO2 concentrations (measured only in the last two years) were higher in the imidacloprid group, 5238±187 ppm, compared to the control group, 4399±170 ppm., suggesting either an increased metabolic activity or a change in within-hive air management. Lower graph: Imidacloprid treatment (red) was associated with higher morning hive weight loss due to forager departure in the first months following treatment (Sept. Oct.) suggesting imidacloprid stimulated bee workers to forage more. Extreme values and days of rain or hive assessments were excluded (see methods), smoothed lines are default ggplot2/R GAMs. Black columns show treatment period. Data are not shown during treatment period because of confounding effects of syrup feeding on hive weight. The low values in 2017 were likely due to poor forage availability that year (see rainfall data in Figure 2).

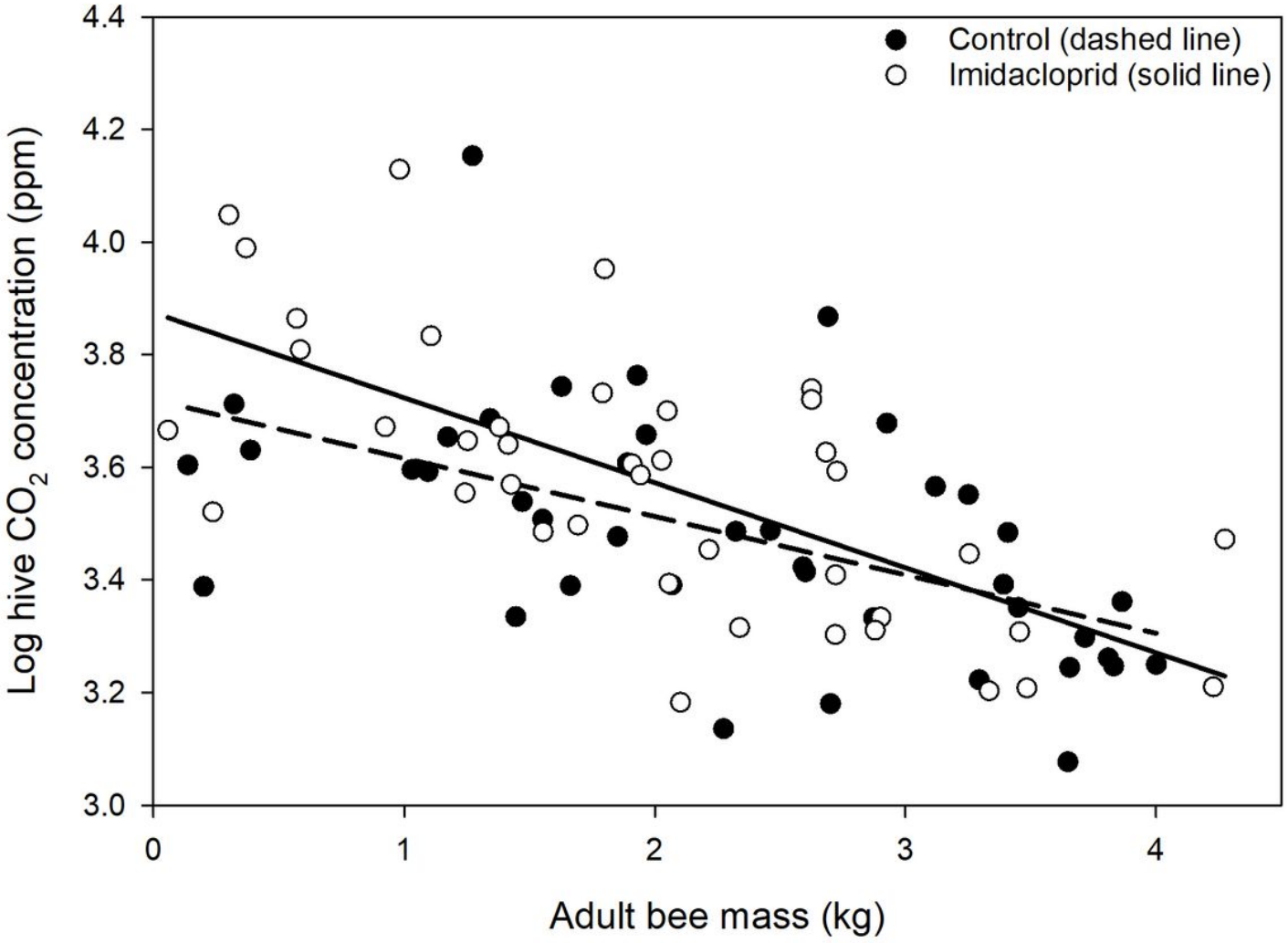

Figure 4

Regression of daily CO2 concentration (ppm), averaged over 3 days just prior to hive evaluations, on adult bee mass (kg). Each point shows data for a given hive at a given hive evaluation; most colonies in these studies were sampled on two or more occasions.

Supplementary Files

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