Field-relevant doses of the systemic insecticide fipronil and fungicide pyraclostrobin impair mandibular and hypopharyngeal glands in nurse honeybees (*Apis mellifera*)

Rodrigo Zaluski1, Luis Antonio Justulin Jr.2 & Ricardo de Oliveira Orsi1

Global decreases in bee populations emphasize the importance of assessing how environmental stressors affect colony maintenance, especially considering the extreme task specialization observed in honeybee societies. Royal jelly, a protein secretion essential to colony nutrition, is produced by nurse honeybees, and development of bee mandibular glands, which comprise a reservoir surrounded by secretory cells and hypopharyngeal glands that are shaped by acini, is directly associated with production of this secretion. Here, we examined individual and combined effects of the systemic fungicide pyraclostrobin and insecticide fipronil in field-relevant doses (850 and 2.5 ppb, respectively) on mandibular and hypopharyngeal glands in nurse honeybees. Six days of pesticide treatment decreased secretory cell height in mandibular glands. When pyraclostrobin and fipronil were combined, the reservoir volume in mandibular glands also decreased. The total number of acini in hypopharyngeal glands was not affected, but pesticide treatment reduced the number of larger acini while increasing smaller acini. These morphological impairments appeared to reduce royal jelly secretion by nurse honeybees and consequently hampered colony maintenance. Overall, pesticide exposure in doses close to those experienced by bees in the field impaired brood-food glands in nurse honeybees, a change that could negatively influence development, survival, and colony maintenance.

Pollination is an indispensable process that ensures ecosystem maintenance, plant reproduction, agriculture, and food security1. Approximately 35% of globally important food crops depend on pollinators2. Among the most common are honeybees (*Apis mellifera* L.), managed worldwide for pollination services1,2 and which generate considerable revenue in the beekeeping industry through their production of honey, pollen, propolis, beeswax, royal jelly, and apitoxin.

Despite the importance of honeybees and other pollinators, their global populations have seen major reductions1,3–5. The main factors responsible are anthropogenic actions that reduce and fragment pollinator habitat, affecting resource availability, the spread of diseases and parasites, and invasive species as well as pesticide exposure. These factors can act alone or synergistically to impair pollinator maintenance5,6.

In particular, existing concerns about pesticide exposure to non-target organisms have raised questions regarding whether field-relevant doses are harmful to honeybees7–9. High pesticide concentrations can immediately kill off colonies10, while chronic exposure to sub-lethal doses, which occurs frequently11–12, may have delayed negative effects13. These sub-lethal effects include compromised resource collection; behavioural changes; decreased longevity, immune function, population growth, reproduction, and learning performance; and the creation of new queens, thus, ultimately influencing colony survival13–21. Specifically, pesticide or xenobiotic exposure is linked to midgut-cell impairment22–26 that can reduce nutrient digestion and absorption in insects27,28 and potentially damage honeybee physiology.

1Núcleo de Ensino, Ciência e Tecnologia em Apicultura Racional (NECTAR), São Paulo State University (UNESP), School of Veterinary Medicine and Animal Science, Department of Animal Production, Botucatu, SP, Brazil. 2São Paulo State University (UNESP), Institute of Biosciences, Department of Morphology, Botucatu, SP, Brazil. Correspondence and requests for materials should be addressed to R.O. (email: orsi@fmvz.unesp.br)
Insecticides are often related to honeybee losses because of their high toxicity and lack of specificity.\textsuperscript{15,20,29} However, pollinators are more likely to encounter fungicides during foraging.\textsuperscript{13} Because fungicides are traditionally considered safe to pollinators, they can be applied on blooming crops, increasing non-target exposure\textsuperscript{13,29} and becoming the most commonly found pesticide in bee colony combs and food stores.\textsuperscript{11,31} Unfortunately, studies show that fungicides do exert a negative effect on bees. Fungicide exposure causes impaired ATP production, increased virus titres, poor brood rearing and queen emergence, population declines, and higher disease susceptibility.\textsuperscript{3,32,33} Fungicide exposure can also cause nutritional deficiencies in colonies, resulting in similar symptoms to those of malnutrition even when pollen is available.\textsuperscript{43}

Honeybees are eusocial insects living in highly organized societies. As ‘super-organisms’, bee colonies are maintained through specialized tasks performed by individuals of a particular caste;\textsuperscript{44} thus, the entire colony is affected if exposure to toxicity hampers a single task.\textsuperscript{35} One of the worker specializations is the nurse, which is characterized by pheromone-stimulated development of mandibular and hypopharyngeal (or brood-food) glands in the head (at ~6 d old).\textsuperscript{37,38} Through the secretion of proteinaceous compounds and pheromones, such as 10-hydroxy-dec-2-enoic acid,\textsuperscript{38,39} these glands, nurse bees produce royal jelly after consuming high quantities of pollen.\textsuperscript{36,37,40} As other colony members have limited pollen-digesting capacity, royal jelly is their main source of protein and is used to feed young larvae, adult bees, and the queen.\textsuperscript{42,43} Thus, a hive’s contact with pesticides would primarily occur through nurse bees that consume contaminated pollen, while the nurses themselves may be more susceptible to those pesticides.\textsuperscript{14,67} However, few reports have characterized the impacts of the combination of different classes of pesticides in brood-food glands of honeybees.

Pyraclostrobin is a strobilurin, which is a group of systemic fungicides that inhibit mitochondrial respiration;\textsuperscript{44} it is widely detected in the pollen of treated crops and in honeybee colonies.\textsuperscript{11,28,50,51} Fipronil is a phenylpyrazole, a systemic neurotoxic insecticide that is widely used in agricultural and veterinary applications. Similar to pyraclostrobin, fipronil is detected in crop pollen and honeybee colonies;\textsuperscript{11,28,50,51} it is also highly toxic to the latter.\textsuperscript{20} Based on existing data, we hypothesized that exposure to these two pesticides in nurse honeybees would promote alterations to mandibular and hypopharyngeal glands, possibly through impairing nutrient digestion and absorption.

To investigate this hypothesis, newly emerged bees were chronically exposed to both pyraclostrobin and fipronil and then brought into the condition of their brood-food glands was evaluated. Mandibular glands comprise a reservoir surrounded by secretory cells that form a pseudo-epithelium, and hypopharyngeal glands are clusters of 8–12 secretory cells connected to the gland’s main channel.\textsuperscript{36} Specifically, we examined secretory-cell height and reservoir volume of the mandibular glands as well as acini size and number in the hypopharyngeal glands. Cell height of the mandibular glands is a main indicator of development and secretory activity of these glands,\textsuperscript{36,52}, whereas acini size in hypopharyngeal glands indicates activity and the amount of royal jelly secreted.\textsuperscript{36,38,40,53,54}

To evaluate the effects of pyraclostrobin and fipronil in doses as close as possible to those to which bees are exposed in the field during pollen collection, we chose a pyraclostrobin dose of 850 ppb, which is between the mean concentrations detected by Petit et al.\textsuperscript{31} and Yoder et al.\textsuperscript{47} in pollen and beebread. For fipronil, we choose a dose of 2.5 ppb, which is close to the mean dose detected in pollen.\textsuperscript{9,50,51}

Our results will provide insight on how damage to brood-food glands in nurse honeybees impacts colony maintenance. Consequently, this study will contribute to efforts aimed at addressing honeybee population declines through improved management of pesticide application.

**Results**

**Individual and combined effects of pyraclostrobin and fipronil on mandibular glands.** In the mandibular glands of pesticide-treated nurses, epithelial secretory cells were significantly decreased in height. Respectively, bees exposed to pyraclostrobin, fipronil, and pyraclostrobin + fipronil saw mean cell-height reduction by 21, 46, and 56%, compared with the control group (Fig. 1). Our results demonstrated that the combination of pesticides on cell height were greater than those produced by exposure to either pesticide alone and indicated an additive effect.

Combined pyraclostrobin + fipronil exposure significantly affected the reservoir area of mandibular glands. Small cross-sectional areas (<499,000 μm²) were more present under combined exposure than in the control, demonstrating an additive effect. Exposure to either pyraclostrobin or fipronil did not result in significantly different reservoir areas from the other two conditions (Fig. 2).

**Individual and combined effects of pyraclostrobin and fipronil on hypopharyngeal glands.** Pesticide exposure did not significantly alter the total number of acini in the hypopharyngeal glands of nurse honeybees (Table 1).

However, when acini were categorized based on area, a more complete view of the changes that occurred could be characterized demonstrating that pesticide-exposed bees had significantly more acini with reduced areas and significantly fewer with larger areas (Kruskal–Wallis one-way ANOVA on ranks, p < 0.001) (Figs 3 and 4). However, the number of acini with reduced areas was not different among pesticide exposure treatments, even when pyraclostrobin and fipronil were combined (Kruskal–Wallis one-way ANOVA on ranks, p > 0.05).

**Colony development.** The areas occupied by worker eggs, uncapped brood, and capped brood in the brood frames did not differ between the treatment groups and the control group (Kruskal–Wallis one-way ANOVA on ranks, p > 0.05) (Supplementary Table S1). Mortality of bees inside or around the hives was not observed.

**Chemical residue analysis and validation.** Pollen patties supplied to colonies were tested to confirm pyraclostrobin and fipronil dosages. No pesticide residues were found in control patties. The average residue level in patties containing pyraclostrobin was 870 ± 53 ppb (mean ± SD). No fipronil residues were detected in
our contaminated patties. We also tested for principal fipronil metabolites (fipronil sulfone, fipronil desulfinyl, fipronil sulfide, and fipronil amide), but none were observed within the limits of detection (LOD = 1.5 ppb). However, given the clear biological changes we observed, fipronil was likely present in pollen patties and had an effect even in concentrations below the LOD.

**Discussion**

Stress-related dysfunction in specialized tasks can affect entire honeybee colonies. Here, we demonstrated that exposure to field-relevant doses of pyraclostrobin and fipronil caused alterations in the mandibular and...
hypopharyngeal glands of nurse honeybees. Specifically, we showed that pesticide exposure reduced epithelial-cell height in mandibular glands, and combined pyraclostrobin + fipronil reduced both cell height and reservoir

| Treatment | Control | Pyraclostrobin | Fipronil | Pyraclostrobin + Fipronil |
|-----------|---------|----------------|----------|----------------------------|
| Total number of acini | 1,230 (1,218–1,340) | 1,279 (1,243–1,310) | 1,290 (1,267–1,352) | 1,290 (1,250–1,319) |

**Table 1.** Total acini count in the hypopharyngeal glands of nurse honeybees. Six days after exposure, total acini were counted in serially collected histological sections taken at 30µm intervals from each honeybee (n = 10). No statistically significant differences were detected (Kruskal–Wallis one-way ANOVA on ranks, p = 0.418). Data are presented as medians and interquartile intervals (Q1–Q3).

**Figure 3.** Cross-sectional areas of acini in hypopharyngeal glands measured in nurse honeybees exposed to treatments of pyraclostrobin and fipronil both individually and combined. Acini area was measured in serially collected histological sections taken at 30µm intervals from each honeybee (n = 10) and then categorized by area. Different letters over bar graphs represent statistically different groups (Kruskal–Wallis one-way ANOVA on ranks with Dunn’s post-hoc test for pairwise comparisons, p < 0.001). All data are represented as the median with interquartile range. *Acini with areas >90,000µm² were not found.

**Figure 4.** Representative histological sections of hypopharyngeal glands in nurse honeybees. (a) Control and (b) pyraclostrobin, (c) fipronil, and (d) pyraclostrobin + fipronil treatments. Note the greater number of acini (emphasized with dotted lines) with reduced area and fewer protein grains in the acini of honeybees exposed to pesticides. Scale bar: 5µm.
volume, indicating an additive effect of these pesticides. In hypopharyngeal glands, while the total number of acini (approximately 550 acini/gland according to previous reports36–38) was unaffected; more acini had reduced areas in bees exposed to pesticides. However, we observed neither additive nor synergistic effects of pyraclostrobin + fipronil on the development of this gland, likely because of the high reduction of acini area as observed in single-pesticide exposure to fipronil or pyraclostrobin. These data corroborate previous studies showing hypopharyngeal-gland impairment in honeybees exposed to pesticides in the laboratory35,55–58 and in bees exposed to pesticides in the field59. As far as we are aware, our study is the first to examine both the mandibular and hypopharyngeal glands in honeybees exposed to pesticides. Given the importance of these brood-food glands to colony nutrition41–43,53, our results newly revealed sub-lethal effects that can affect colony maintenance.

The ratio of mandibular to hypopharyngeal gland secretions deposited around each larva reared in the colony varies depending on larval age, sex, and caste40,41. Queen larvae receive only mandibular-gland secretions during the first 3 d, and afterwards receive secretions produced by both the mandibular and hypopharyngeal glands in a 1:1 ratio40. Worker larvae are fed secretions from both the mandibular and hypopharyngeal glands in ratios of 1:3 to 1:440, which are mixed with pollen after the third day63. Thus, nurse bees without mandibular glands have difficulty rearing queens64. Indeed, our work suggests that pesticide-related impairments to both studied glands in nurses resembles the effects of poor nutrition on the colony and brood-food gland development53,56. Studies have demonstrated that restricting worker access to nutrients during development causes physical and behavioural problems in adult honeybees65. Pesticide-linked alterations to brood-food glands can harm brood care through a reduction in royal jelly quantity/quality, which can threaten caste differentiation, bee development, and colony maintenance, all contributors to high colony losses worldwide66,67,68. We highlight that royal jelly production is related to the development of mandibular and hypopharyngeal glands of nurse honeybees69. We emphasize the importance of direct royal jelly measurement in colonies exposed to pesticides in future studies to evaluate its quantity and quality.

Our study provides additional explanations for why exposure to sub-lethal doses of pesticides can reduce colony growth and queen production18 while increasing queen supersedeure60. Queens reared in colonies exposed to pesticides have lower body and ovary weight, if they survive at all71. Pesticide studies similar to ours have suggested that compromised immunity in developing queens may be the underlying cause of these symptoms32. Here, we hypothesized that nutritional deficits from low-quality/quantity royal jelly produced by nurses with impaired glands would likely contribute to the negative effects of pesticides on queens.

Furthermore, nurse honeybees with hampered hypopharyngeal gland development appear to become foragers more rapidly60. Premature foraging activity in honeybees is associated with a reduction in the number of nurses, insufficient brood rearing, and a reduced lifespan, all of which can accelerate colony collapse68,72. If this is the case, then the morphological impairments we observed in both the hypopharyngeal and mandibular glands may cause even faster nurse-to-forager changeover, meaning that the colony is fed low-quality royal jelly over a short period.

All honeybees used in our experiments were from the same colonies, reared under the same conditions, and received the same pollen diet. Through the inclusion of pollen and brood pheromones, this experimental design controlled for potential confounding factors that could hamper the full development of brood-food glands even in the pesticide treatments55,60. Given that the bees were provided with a generally favourable environment with sufficient food resources, we hypothesize that pesticides reduced nutrient digestion and absorption in nurses, contributing to improper glandular development. Indeed, such effects have been reported in insects27–29,30 with studies demonstrating that exposure to pesticides affect the honeybee midgut31–33, including some that demonstrate fipronil induces death of digestive midgut epithelial cells31. Further studies are necessary to explore the connection between nutrition and pesticides exposure and the glandular development of bees in greater detail.

In addition, the effects of diet quality on bees exposed to pesticides need be considered, because this factor influences hypopharyngeal gland development in nurse bees according to Renzi et al.37.

In our study, we focused on two pesticides that can be stored in colonies for long periods and across a range of doses. Our chemical residue analysis of pollen patties confirmed the presence of pyraclostrobin residues within or below the amounts detected in various pollens (mean: 2,787.1 ± 1,890.1 ppb31; mean: 10,458 and 267 ppb49) and in bee bread (range: 319–2,170 ppb47). Thus, our dose of pyraclostrobin substantiated a realistic exposure concentration of this fungicide that bees would encounter. Based on studies that have calculated the total amount of pollen consumed by nurse honeybees44, if a nurse bee were to feed on the pyraclostrobin–contaminated pollen patties that were detected in our study (mean 870 ppb), it could consume up to a maximum of 10,440 pg of pyraclostrobin within one day of intensive feeding and a mean total amount of 33,930 pg in six days.

We highlight that the fipronil dose that was chosen for bee exposure in this study is within the range of field-relevant doses of this insecticide that have been detected in pollen (mean: 2.3–2.8 ppb39,50,51) and is close to rates broadly applied for application in fields49, which implies that the exposure of bees to this insecticide in the field is similar to the dose used in our study. The dose of fipronil added to pollen patties in our study (2.5 ppb) could result in nurse exposure up to a maximum of 30.0 pg of fipronil within one day of intensive feeding and a total of 97.5 pg in six days, according to one estimate of daily pollen consumption by nurse honeybees44. However, observed fipronil residue levels in our patties were lower than we expected. The low-field-relevant dose of fipronil that we chose for this study and its probable degradation may have reduced the quantity of fipronil and generated low levels of metabolites that preclude their detection by UHPLC/MS-MS within the LOD in this study. Despite low fipronil concentrations, the contaminated patties caused significant biological alterations in honeybee glands. Thus, residual fipronil and related metabolites were clearly present and had an effect even at doses below the LOD (1.5 ppb) and may be close to the amount of residue detected by Chauzat et al.73 in pollen (fipronil mean content: 1.2 ppb; metabolite mean content: 1.0–1.7 ppb). The effect of fipronil, even at very low concentrations, impaired the brood-food glands of nurse bees, and similar results have been reported for hypopharyngeal glands by Hatjina et al.36 and Heylen et al.38 for bees exposed to the insecticide imidacloprid at sub-lethal doses.
Most studies that found fungicides like pyraclostrobin in pollen and other bee products have not investigated fipronil and their metabolites, but primarily included neonicotinoids, which also have deleterious effects on the hypopharyngeal glands of nurse honeybees. Mullan et al. detected pyraclostrobin and fipronil in bee hives. The highest concentrations of pyraclostrobin detected in wax, pollen, and bees were 438.0, 265.0, and 9.0 ppb, respectively, while mean detection of fipronil in these samples was 0.2, 0.1, and 21.9 ppb, respectively. It is important to highlight the persistence of fipronil and pyraclostrobin in the environment owing to their systemic properties. The fact that bees can collect and store contaminated resources in their hives, and the increase in pesticide detection in migration colonies can increase bee exposure to pesticides such as those used in our study.

In our study, we choose to work with commercial formulations of pyraclostrobin and fipronil as these are more environmentally relevant and represent a better approximation of the occurrence of bee poisoning in the fields. Some inert ingredients (i.e. builder, preservative, filler, adjuvants, or stabilizer components) can also have toxic effects on honeybees and contribute to the declining health of their populations. A study performed by Ciarlo et al. demonstrated that ingestion of spray adjuvants impairs learning performance of honeybees, and Zhu et al. confirmed that inert ingredients can be highly toxic to developing honey bees; however, we know of no studies comparing the effects of inert ingredients, active ingredients, and commercial formulations of pesticides on mandibular and hypopharyngeal gland development in honeybees. A full-label disclosure of the composition of pesticides, including inert ingredients, is essential and can enable a more appropriate risk assessment of these substances to pollinators and non-target species.

Although a reduction in colony development was not observed in our study, probably because of the short exposure period and low doses of pesticides used, we highlight that impacts that are not detectable at the colony level are still impacting the individual bees. Our results provide evidence that pesticide exposure over a short period (six days) results in clear changes to the mandibular and hypopharyngeal glands of nurse honeybees. Because honeybees can access contaminated and non-contaminated pollen in the field, the deleterious effects may not be as obvious as what we observed. However, honeybees can be exposed to multiple pesticides for a far longer period in the field, with a greater probability of interactions between pesticides. Thus, our results may understate the actual negative impact of pesticide exposure on nurse bees.

Detecting deleterious effects of pesticides in doses that are experienced by pollinators in the field is a critical step toward avoiding the negative impacts that these substances may have on bee maintenance and is crucial in terms of prohibition or re-registration of pesticides. In conclusion, our data highlight the importance of subjecting pesticides to tests that are more rigorous so that sub-lethal effects on important insects like honeybees can be detected. Our results can contribute to the development of policies aimed at promoting more appropriate pesticide risk assessments, mainly at doses close to those that are found in resources collected by bees and to the adoption of management responses aimed at reducing global pesticide application (with emphasis on highly toxic insecticides such as fipronil) to protect pollinators. Because of the negative effects of the fungicide pyraclostrobin on brood food glands, we also highlight the importance of evaluating other molecules of this class that are traditionally considered safe to pollinators and the importance of avoiding spraying blooming crops with them.

Methods

Test subjects were Africanized Apis mellifera kept in the apiary of the Faculty of Veterinary Medicine and Animal Science, UNESP, Botucatu, São Paulo, Brazil, under normal living conditions. All colonies were free of diseases and parasites; thus, no treatment against Varroa destructor was performed.

Experimental design, diet supply, nurse bee collection, and residue analysis. Experiments were performed during March–May 2016. Two brood combs of emerging bees from five donor colonies were removed, marked, and placed in separate, incubated cages under conditions of 34 °C and 80% relative humidity. The thoraces of newly emerged bees (<10 h) of each donor colony were marked with a specific colour using a non-toxic paint (Posca Paint Pens, Mitsubishi Pencil, Japan) to distinguish colony origin. After marking, we randomly distributed approximately 40 bees of each donor colony to the experimental colonies where they received contaminated food.

Each experimental colony was housed in a nucleus with two sealed brood frames, two open brood frames, one egg-laying frame, and approximately 4,000 bees. The queens in each experimental nucleus were sisters, naturally mated and at four months of age. All frames with pollen/beebread were removed from experimental nuclei 24 h before marked bees were introduced. Pollen traps were installed at the entrances to maximize the amount of contaminated diet consumed. Honey syrup (50% w/v) was supplied ad libitum to all colonies.

The areas (cm²) occupied by worker eggs, capped brood, and uncapped brood in all frames in the nucleus were measured. Measurements were performed at the beginning of the study when food was supplied and every 2 weeks, for a period of 75 days.

All pollen and honey used in this study were organic and polyfloral (Breyer & Cia Ltda, União da Vitória, Brazil) to avoid pesticide cross-contamination. Stock pyraclostrobin and fipronil solutions were prepared from formulated products (Comet® 250 g a.i. L−1, inert ingredients 802 g L−1, BASF Schwarzheide GmbH, Schwarzheide, Germany; Regent 800WG® 800 g a.i. kg−1, inert ingredients 200 g kg−1 BASF Agri-Production SAS, Saint Aubin Les Elbeuf, France). Compositions of inert ingredients were not disclosed on the labels for pesticides used in this study. All commercial pesticides were diluted in distilled water. The final concentration of each treatment dose (pyraclostrobin: 850 ppb; fipronil: 2.5 ppb; pyraclostrobin + fipronil: 850 ppb + 2.5 ppb) was obtained through adding concentrated pyraclostrobin and fipronil solutions to honey syrup (50% w/v). This mixture was added to pollen powder in a 3:1 (pollen to honey syrup) ratio. Pollen patties were homogenized, portioned in cellophane paper (100 g), and stored in a freezer (−20 °C) until use. Control pollen patties without pesticides were prepared following the same procedure.
Residue analysis was performed on two subsamples (50 g) of each pollen patty, based on the QuEChERS (quick, easy, cheap, effective, rugged, and safe) pesticide extraction method. The QuEChERS citrate-buffering version (European Standard EN 15662) was modified based on that of a previous study developed for pesticide and antibiotic residue determination in honey samples. Ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) analysis of the residues was performed at the Laboratory of Pesticide Residue Analysis (LARP), Chemistry Department, Federal University of Santa Maria, Santa Maria, Brazil. Pesticide standard materials with purity >99% were purchased from Dr. Ehrenstorfer (Germany). To ensure quality and comparability of results, pesticide residues in pollen patties were validated according to the methods of the Brazilian Health Surveillance Agency Legislation. Method selectivity was assessed by analysing blank samples and was checked for any interference around the retention time of the target analytes; no interfering compounds were observed by UHPLC-MS/MS. Linearity, calculated based on the spike levels for each analysed compound, resulted in satisfactory calibration curves in the range 0.1–200 µg kg⁻¹ with determination coefficients (r²) higher than 0.99 for all compounds and a relative standard deviation below 20%.

Chromatographic separation was performed with an Acquity BEH C18 column (50 × 2.1 mm; 1.7 µm particle size) from Waters (Milford, USA) maintained at 45°C. Mass spectrometry (MS) analyses were performed using the selected reaction monitoring mode. All MS parameters were optimized under the electrospray ionization (+/−) mode. Because fipronil was not detected initially, its principal metabolites (fipronil sulfone, fipronil desulfinyl, fipronil sulfide, and fipronil amide) were analysed. The LOD was 1.5 ppb for pyraclostrobin, fipronil, and all fipronil metabolites.

Each experimental colony received one contaminated pollen patty daily (for 6 d), placed on the top bars of frames, and the amount acquired by the colony was measured by weighing the food not consumed. This measure ensured that marked honeybees consumed contaminated pollen ad libitum from the first day until nursing, when they produce more royal jelly. The amount of pollen patties acquired daily by the colonies did not differ between the treatment group and the control with an average daily amount consumed of 80 ± 6 g (mean ± SD) among all colonies (Kruskal–Wallis one-way ANOVA on ranks, p = 0.328). Ten marked nurse honeybees (two per donor colony, distinguished according to their colour) were collected in each experimental colony on day 7 (after 6 d of exposure to the contaminated diet). Bees were anesthetized in a freezer and then decapitated.

Processing of material for morphological analysis. To increase exoskeleton permeability, entire heads were fixed (4% paraformaldehyde/1 × PBS) for 24 h, washed with distilled water, and transferred to a solution containing sodium hypochlorite (5%) and sodium hydroxide (7.5%) for 5 h at room temperature (25 ± 2°C). Heads were dehydrated using an ascending ethanol series (70, 80, 90, and 95%; 2 h per bath). Subsequently, a cut was made in the anterior and posterior portions of the heads (eliminating about 1 mm of chitin) under a stereomicroscope. The heads were embedded in resin (Leica Historesin Embedding Kit; Leica, Nussloch, Germany) and sliced with a microtome (Leica RM2155; Germany); frontal cross-sections were 3 µm and separated by 30 µm. Histological sections were made every 30 µm to avoid measuring acini from the same hypopharyngeal gland. The total area of the mandibular glands (one pair cross-sectioned per animal) was determined from 20 histological sections using 400 measurements in total and then, the glands were classified according to their size (<499,000; 500,000–1,000,000; and >1,000,000 µm²) for statistical analyses to evaluate if a change in the area influenced the reservoir volume of these glands.

For the hypopharyngeal glands, the number and area of acini were measured in all histological sections where they were present. Considering that acini area is the main indicator of hypopharyngeal gland activity and royal jelly secretion, acini area per bee (n = 10) for a total of 1,000 cell measurements; all glands and cells were randomly selected. Analyses were performed on mean cell height per measured gland. The total area of the mandibular glands (one pair cross-sectioned per animal) was determined from 20 histological sections using 400 measurements in total and then, the glands were classified according to their size (<29,999; 30,000–59,999; 60,000–89,999; and >90,000 µm²) for statistical analyses. This standardized histological method to analyse acini area is less influenced by evaluation bias than is visual assessment of acini size (e.g. choice of acini, ability to extract and measure them) and thus, is more appropriate for measuring acini that display an irregular form.

Statistical analyses. Measurement data were first tested for normality and homogeneity of variance by Kolmogorov–Smirnov and Levene’s test, respectively. Data were compared by Kruskal–Wallis one-way ANOVA on ranks and Dunn’s post-hoc tests for pairwise comparisons of all groups using SigmaStat (version 3.5; Systat Software Inc., San Jose, CA). Significance was set at p < 0.05.

Data availability. The datasets generated and/or analysed in the current study are available from the corresponding author on reasonable request.

References
1. Potts, S. G. et al. Safeguarding pollinators and their values to human well-being. *Nature* 540, 220–229 (2016).
2. Klein, A. et al. Importance of pollinators in changing landscapes for world crops. *Proc. Roy. Soc. B-Biol. Sci.* 274, 303–313 (2007).
3. Johnson, R. M., Ellis, M. D., Mullin, C. A. & Frazier, M. Pesticides and honey bee toxicity – USA. *Apidologie* 41, 312–331 (2010).
4. vanEngelsdorp, D. & Meixner, M. D. A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *J. Invertebr. Pathol.* 103, S80–S95 (2010).
47. Yoder, J. A.

46. Ivice, D. Curative and eradicative effects of fungicides in beekeeping. (ed. Carisse, O.) 1–22 (InTech, 2010).

45. Halm, M. P., Rortais, A., Arnold, G., Taséi, J. N. & Rault, S. New risk assessment approach for systemic insecticides: the case of honey bee health. Proc. Nat. Acad. Sci. USA 110, 18466–18471 (2013).

44. Rortais, A., Arnold, G., Halm, M. P. & Touffet-Briens, F. Modes of honey bees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees. Nature 491, 105–108 (2012).

43. Henry, M. et al. A common pesticide decreases foraging success and survival in honey bees. Science 336, 348–350 (2012).

42. Yang, E. C., Chang, H. C., Wu, W. Y. & Chen, Y. W. Impaired olfactory associative behavior of honeybee workers due to contamination of imidacloprid in the larval stage. Plos One 7, e94772 (2012).

41. Whitehorn, P. R., O’Connor, S., Wackers, F. L. & Goulson, D. Neonicotinoid pesticide reduces bumble bee colony growth and queen production. Science 336, 351–352 (2012).

40. Di Prisco, G. et al. Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bee. Plos One 6, e23108 (2010).

39. Zaluski, R., Kadri, S. M., Alonso, D. P., Ribolla, P. E. M. & Orsi, R. O. Fipronil promotes motor and behavioral changes in honey bees (Apis mellifera) and affects the development of colonies exposed to sublethal doses. Environ. Toxicol. Chem. 34, 1062–1069 (2015).

38. Wu-Smart, J. & Spivak, M. Sub-lethal effects of dietary neonicotinoid insecticide exposure on honey bee queen fecundity and colony development. Sci. Rep. 6, 32108, doi:10.1038/srep32108 (2016).

37. Gregorc, A. & Bowen, I. D. Histochromatological characterization of cell death in honeybee larvae midgut after treatment with Paenibacillus larvae, amitraz and oxytetracycline. Cell Biol. Internat. 24, 319–324 (2000).

36. Forkphah, C., Dixon, L. R., Fahrbach, S. E. & Rueppell, O. Xenobiotic effects on intestinal stem cell proliferation in adult honey bee (Apis mellifera L) workers. Plos One 9, e91180 (2014).

35. Oliveira, R. A., Roat, T. C., Carvalho, S. M. & Malaspina, O. Side-effects of thiamethoxam on the brain and midgut of the Africanized honey bee (Apis mellifera (Hymenoptera: Apidae). Environ. Toxicol. 29, 1122–1133 (2014).

34. Gregorc, A. & Ellis, J. D. Cell death localization in situ in laboratory reared honey bee (Apis mellifera L) larvae treated with pesticides. Pestic. Biochem. Physiol. 99, 208–207 (2011).

33. da Silva Cruz, A., da Silva-Zacarin, E. C., Bueno, O. C. & Malaspina, O. Morphological alterations induced by boracic acid and fipronil in the midgut of worker honeybee (Apis mellifera L.) larvae: morphological alterations in the midgut of A. mellifera L. Cell Biol. Toxicol. 26, 165–176 (2010).

32. Dow, J. A. T. Insect midgut function. Adv. Insect Physiol. 19, 187–328 (1987).

31. Chown, S. & Nicolson, S. W. Insect Physiological Ecology: Mechanisms and Patterns. (Oxford University Press, 2004).

30. Stover, K. A. & Eitzer, B. D. Using a hazard quotient to evaluate pesticide residues detected in pollen trapped from honey bees (Apis mellifera) in Connecticut. Plos One 8, e77550 (2013).

29. Yoshimura, M. A., Luo, Y., Ma, Z. & Michalildies, T. J. Sensitivity of Monilinia fructicola from stone fruit to thiophanemethyl, iprodione, and tebuconazole. Plant Dis. 88, 373–378 (2004).

28. Pettis, J. S. et al. Crop pollination exposes honey bees to pesticides which alters their susceptibility to the gut pathogen Nosema ceranae. Plos One 8, e70182 (2013).

27. DeGrandi-Hoffman, G., Chen, Y. & Simonds, R. The effects of pesticides on queen rearing and virus titer in honey bees (Apis mellifera L.). Insects 4, 71–89 (2013).

26. Simon-Delso, N. Crop pollination exposure to neonicotinoid and fungicide residues in pollen and single bumblebees using a scaled down QuEChERS method for exposure assessment. Anal. Bioanal. Chem. 407, 8151–8162 (2015).

25. Hrassnigg, N. & Crailsheim, K. Adaptation of hypopharyngeal gland development to the brood status of honeybee (Apis mellifera L.) colonies. J. Insect Physiol. 44, 929–939 (1998).

24. Crailsheim, K. Trophallactic interactions in the adult honey bee (Apis mellifera L.). Apidologie 29, 97–112 (1998).

23. Michener C. D. The Social Behavior of the Honey Bee. (Harvard University Press, 1974).

22. Crailsheim, K. The flow of jelly within a honeybee colony. J. Comp. Physiol. B 162, 681–689 (1992).

21. Rortais, A., Mold, G., Halm, M. P. & Touffet-Briens, F. Modes of honeybees exposure to systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by different categories of bees. Apidologie 36, 71–83 (2005).

20. Halm, M. P., Rortais, A., Arnold, G., Tsai, J. N. & Rault, S. New risk assessment approach for systemic insecticides: the case of honey bees and imidacloprid (Gaucho). Environ. Sci. Technol. 40, 2448–2454 (2006).

19. Ivice, D. Curative and eradicative effects of fungicides in Fungicides (ed. Carisse, O.) 1–22 (InTech, 2010).

18. Yoder, J. A. et al. Fungicide contamination reduces beneficial fungi in bee bread based on an area-wide field study in honey bee, Apis mellifera, colonies. J. Toxicol. Environ. Health. A 76, 587–600 (2013).

17. David, A., Botías, C., Abdul-Sada, A., Goulson, D. & Hill, E. M. Sensitive determination of mixtures of neonicotinoid and fungicide residues in pollen and single bumblebees using a scaled down QuEChERS method for exposure assessment. Anal. Bioanal. Chem. 407, 8151–8162 (2015).
49. Fraizer, M. T. et al. Assessing honey bee (Hymenoptera: Apidae) foraging populations and the potential impact of pesticides on eight U.S. crops. J. Econ. Entomol. 108, 2141–2152 (2015).
50. European Food Safety Authority. Conclusion on the peer review of the pesticide risk assessment for bees for the active substance fipronil. EFSA J. 3158, 1–51 (2013).
51. Bernal, J. et al. Overview of pesticide residues in stored pollen and their potential effect on bee colony (Apis mellifera) losses in Spain. J. Econ. Entomol. 103, 1964–1971 (2010).
52. Santos, D. S., Souza, E. A., Vieira, C. U., Zanuncio, J. C. & Serrão, J. E. Morphology of mandibular and intramandibular glands in workers and virgin queens of Melipona scutellaris. Apidologie 46, 23–34 (2015).
53. Knecht, D. & Kaatz, H. H. Patterns of larval food-production by hypopharyngeal glands in adult worker honey-bees. Apidologie 21, 457–468 (1990).
54. Raliman, S., Thangkhiew, I. & Hajong, S. R. Hypopharyngeal gland activity in task-specific workers under brood and broodless conditions in Apis cerana indica (Fab.). J. Apic. Sci. 58, 61–70 (2014).
55. Smođić Sker, M. I. & Gregorc, A. Heat shock proteins and cell death in situ localisation in hypopharyngeal glands of honeybee (Apis mellifera carnica) workers after imidacloprid or coumaphos treatment. Apidologie 41, 73–86 (2010).
56. Hatjina, F. et al. Sublethal doses of imidacloprid decreased size of hypopharyngeal glands and respiratory rhythm of honeybees in vivo. Apidologie 44, 467–480 (2013).
57. Renz, M. T. et al. Combined effect of pollen quality and thiamethoxam on hypopharyngeal gland development and protein content in Apis mellifera. Apidologie 47, 779–788 (2016).
58. De Smet, L. et al. Stress indicator gene expression profiles, colony dynamics and tissue development of honey bees exposed to sub-lethal doses of imidacloprid in laboratory and field experiments. Plos One 12, e0171529 (2017).
59. Böhme, F., Bischoff, G., Žebita, C. P., Rosenkrantz, F. & Wallner, K. Chronic exposure of honeybees, Apis mellifera (Hymenoptera: Apidae), to a pesticide mixture in realistic field exposure rates. Apidologie 48, 353–363 (2017).
60. Beetsma, J. The process of queen-worker differentiation in the honeybee. Bee World 60, 24–39 (1979).
61. Brouwers, E. V. M., Ebert, R. & Beetsma, J. Behavioural and physiological aspects of nurse bees in relation to the composition of larval food during caste differentiation in the honeybee. J. Apic. Res. 26, 11–23 (1987).
62. Jung-Hoffmann, I. Die determination von königin und arbeiterin der honigbiene. Z. Bienenforsch. 8, 296–322 (1966).
63. Haydak, M. H. Honey bee nutrition. Annu. Rev. Entomol. 15, 143–156 (1970).
64. Peng, Y. S. & Jay, S. C. Larval rearing by worker honey bees lacking their mandibular glands II: rearing by larger numbers of worker bees. Can. Ent. 111, 101–104 (1979).
65. Degrandi-Hoffman, G., Chen, Y. & Huang, E. & Huang, M. H. The effect of diet on protein concentration, hypopharyngeal gland development and virus load in worker honey bees (Apis mellifera L.). J. Insect Physiol. 56, 1184–1191 (2010).
66. Scefield, H. N. & Mattila, H. R. Honey bee workers that are pollen stressed as larvae become poor foragers and waggle dancers as adults. Plos One 10, e0121731 (2015).
67. Lee, K. V. et al. A national survey of managed honey bee 2013–2014 annual colony losses in the USA. Apidologie 46, 292–305 (2015).
68. Seitz, N. et al. A national survey of managed honey bee 2014–2015 annual colony losses in the USA. J. Apic. Res. 54, 292–304 (2016).
69. Bovi, T. S., Onari, P., Santos, S. A. A., Justulin, L. A. & Orsi, R. O. Apitoxin harvest impairs hypopharyngeal gland structure in Apis mellifera honey bees. Apidologie doi: https://doi.org/10.1007/s13592-017-0520-8 (2017).
70. Sandrock, C. et al. Impact of chronic neonicotinoid exposure on honeybee colony performance and queen supersEDURE. Plos One 9, e103592 (2014).
71. Haarmann, T., Spivak, M., Weaver, D., Weaver, B. & Glenn, T. Effects of Dufulance and coumaphos on queen honey bees (Hymenoptera: Apidae) in two commercial queen rearing operations. J. Econ. Entomol. 95, 28–35 (2002).
72. Khoury, D. S., Myerscough, M. R. & Barron, A. B. A quantitative model of honey bee colony population dynamics. Plos One 6, e18491 (2011).
73. Chauzat, M.-P. et al. An assessment of honeybee colony matrices, Apis mellifera (Hymenoptera: Apidae) to monitor pesticide presence in continental France. Environ. Toxicol. Chem. 30, 103–111 (2011).
74. Mullin, C. A., Fine, J. D., Reynolds, R. D. & Fraizer, M. T. Toxicological risks of agrochemical spray adjuvants: organosilicone surfactants may not be safe. Front. Public Health. 4, 92 (2016).
75. Carlo, T. I., Mullin, C. A., Fraizer, J. L. & Schmehl, D. R. Learning impairment in honey bees caused by agricultural spray adjuvants. Plos One 7, e40848 (2012).
76. Zhu, W., Schmehl, D. R., Mullin, C. A. & Fraizer, J. L. Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. Plos One 9, e77347 (2014).
77. Anastassiades, M., Lehotay, S., Stainbacher, D. & Schenk, F. J. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and “dispersive solid-phase extraction” for the determination of pesticide residues in produce. J. AOAC Int. 83, 412 (2000).
78. European Standard Method EN 15662. Foods of plant origin – determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE (QuEChERS method). http://www.cen.eu (2008).
79. Orso, D. et al. Simultaneous determination of multiclass pesticides and antibiotics in honey samples based on ultra-high performance liquid chromatography-tandem mass spectrometry. Food Anal. Method 9, 1638–1653 (2016).
80. Brazilian Health Surveillance Agency (ANVISA). Guia para validação de métodos analíticos e bioanalíticos. http://portal.anvisa.gov.br/documents/33880/2560070/RE_899_2003.pdf/1077d22-ab7f-473c-ab4e-6827dd5f4d42 (2003).

Acknowledgements
This study was supported by the Brazilian Council for Scientific and Technological Development/CNPq (grant number: 165696/2014-1).

Author Contributions
R.Z. and R.O.O. designed and performed the experiments. R.Z. and L.A.J.J. performed morphological analyses. R.Z., R.O.O. and L.A.J.J. performed the analyses. R.Z. wrote the paper. All authors reviewed the manuscript.

Additional Information
Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-017-15581-5.

Competing Interests: The authors declare that they have no competing interests.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
