Xanthoxylin Found In Secretions Of Epormenis Cestri Feeding On Sebastiania Schottiana Trees Causes Mass Death Of Honeybee Apis Mellifera Larvae

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Research Article

Keywords: River disease, NMR metabolomics, GSMS metabolomics, honeydew honey, toxic excretions

DOI: https://doi.org/10.21203/rs.3.rs-408843/v1

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Abstract

The "River Disease" (RD), a disorder impacting honeybee colonies located close to waterways with abundant riparian vegetation (including Sebastiania schottiana, Euphorbiaceae), kills newly hatched larvae. Forager bees from RD-affected colonies collect honeydew excretions from Epormenis cestri (Hemiptera: Flatidae), a planthopper feeding on trees of S. schottiana. First-instar honeybee larvae fed with this honeydew died. Thus, we postulated that the nectars of RD-affected colonies had a natural toxin coming from either E. cestri or S. schottiana. An untargeted metabolomics characterization of fresh nectars extracts from colonies with and without RD allowed to pinpoint xanthoxylin as one of the chemicals present in higher amounts in nectar from RD-affected colonies than in nectars from healthy colonies. Besides, xanthoxylin was also found in the aerial parts of S. schottiana and in the honeydew excreted by E. cestri feeding on this tree. A larva feeding assay where xanthoxylin-enriched diets were offered to 1st instar larvae showed that larvae died in the same proportion as larvae did when offered enriched diets with nectars from RD-colonies. These findings demonstrate that a xenobiotic can mimic the RD syndrome in honeybee larvae and provide evidence of an interspecific flow of xanthoxylin among three trophic levels. Further, our results give information that can be considered when implementing measures to control this honeybee disease.

Introduction

To fulfill their nutritional requirements, honeybees (Apis mellifera) depend mostly on nectar and pollen from flowers (Michener 1974). While foraging, honeybees collect basic nutrients from the primary metabolism (proteins and carbohydrates) but also, they come across and accidentally gather secondary metabolites that plants biosynthesize for different ecological purposes (Adler 2000; Bankova et al. 2018; Detzel and Wink 1993). Honeybees also collect honeydew secretions of different plant parts (CX 12-1981 2019); especially when this supply is abundant, as it is in the case of bracatinga (Mimosa scabrella) (Bergamo et al. 2019), oak honeydew (Quercus pirenaica) and evergreen (Quercus ilex) (Seijo et al. 2019) and pine (Pinus brutia) (Özkök et al. 2018). Besides, in times of shortage of floral nectar honeybees also collect other resources such as honeydew excretions from plant sucking insects that are carbohydrate rich (CX 12-1981 2019; Simova et al. 2012).

The "River disease" (RD) is a disorder that kills newly hatched larvae in honeybee colonies located closed to fresh waterways with abundant riparian vegetation (Invernizzi et al. 2018). It usually appears at the end of spring and can lead to massive larval losses for 40–50 days. Affected colonies begin to depopulate due to the lack of replacement of dead bees and significant colony losses can occur if this situation is not reverted (Invernizzi et al. 2018). Forager bees from RD-affected colonies exhibited high activity on trees of Sebastiania schottiana (Euphorbiaceae) infested with Epormenis cestri (Hemiptera: Flatidae) (Invernizzi et al. 2018; Santos and Invernizzi 2020). Epormenis cestri feeding on these trees secrete sugar-rich honeydew that attracts bees. The planthopper honeydew collected by the honeybees is then turned into honeydew honey within the colony. In our previous work, we observed that larvae fed with this honeydew die in their first days of life (Invernizzi et al. 2018). Besides, ingestion of this toxic honeydew by larvae triggers alterations of the localization and the immunoexpression of caspase-3 in fat body cells, suggesting a deregulation of the apoptosis that affect the normal larval development (Viotti et al. 2021). Therefore, we hypothesized that the honeydew nectar of RD-affected colonies had a natural toxin coming from either E. cestri or S. schottiana. To address if plant or planthopper chemicals are responsible for the mortality of honeybee larvae in RD-affected colonies, an untargeted metabolomics approach (Roessner and Dias 2013) in combination with a larvae feeding assay was used. We collected fresh nectars from colonies with and without RD. (In this work, to simplify, samples from open cells from whatever colony will be denominated nectars, although the origin of the samples may differ in healthy and RD-affected colonies. RD nectars care indeed
a mixture of floral nectars and honeydew excretions.) The results of the analyses by nuclear magnetic resonance spectroscopy (NMR) and gas chromatography coupled to mass spectrometry (GCMS) of nectar extracts were compared by means of multivariate analyses to mark out substances present (or in higher concentrations) in RD-affected colonies and absent (or in lower concentrations) in RD-free colonies (from now on these colonies will be named "healthy" colonies). Besides, to detect the origin of the pinpointed chemicals in nectars, aerial parts of *S. schottiana* and honeydew from *E. cestri* were also collected and analyzed. Our main goal was then identifying the substances that kill the larvae in RD-affected colonies and expanding the knowledge of this recently described disease of the honeybee brood which can strongly impact on the beekeeping industry in Uruguay and the region where this syndrome occurs.

**Methods And Materials**

**Chemicals and Solvents.** Pure compounds (2′-Hydroxy-4′,6′-dimethoxyacetophenone -xanthoxylin- and the remaining chemicals) were obtained from Sigma-Aldrich; deuterated solvents from Cambridge Isotope Laboratories, Inc. and the remaining solvents from Mallinckrodt, Inc.

**Biological Material.** Nectar samples were collected from uncapped cells from colonies (with and without RD) from different locations in Uruguay (N = 18/group). RD-affected colonies were located near Río Yi and Arroyo Maciel and were from the same apiary studied in our previous report (Invernizzi et al. 2018). Except for eggs, these colonies had a total absence of brood which is the most obvious symptom of RD-affected colonies (Invernizzi et al. 2018). Healthy colonies were located at different regions of the country (for the complete list of localities see Supplementary material Table S1).

The aerial parts of *S. schottiana* were collected in December of 2017 at a riverbank nearby Arroyo Maciel, Durazno, Uruguay (-33,390578° S, -56.6107782° W). The species was identified by César Fagündez Pachón from Centro Universitario Regional Este, UDELAR. Plant material was air-dried and then ground using a mill (IKA Werke MF 10) before extractions.

*Epormenis cestri* secretions were obtained by placing pre-washed glass plates (20 x 15 cm) for 12 h, 20 cm under *E. cestri* groups feeding on *S. schottiana* trees located in the same place where plant material was collected. Once gathered, glass plates were kept at -4°C until extraction.

Worker honeybee larvae of a healthy colony (hybrid of *A. mellifera mellifera*, *A. mellifera scutellata* and *A. mellifera ligustica*) were used in the larval bioassay (see below).

**Extractions of Biological Material for GCMS Analyses.** Nectars (1 g of each sample) from colonies with and without RD were extracted consecutively with hexane and dichloromethane (DCM). For each solvent 3 extractions were done with 5 mL. Extracts were analyzed by GCMS in the TIC (total ion chromatogram) and SIM (single ion monitoring) modes (see below). Extracts from insect secretions were produced from the D$_2$O-insoluble matter obtained from glass pieces (see below) which was partitioned between DCM and water (4 times, 0.3 mL, under sonication for 4 min). These DCM extracts were dried on anhydrous CuSO$_4$, filtered concentrated and then analyzed by GCMS in the SIM mode. In the case of plant material, hexane and dichloromethane (DCM) extracts were obtained from the dried residue of methanol (90%, v/v) extracts performed by maceration of 2 Kg (rT, 5 days, 2 consecutive extraction with 5 L (Yunes et al. 1990). This dried residue was consecutively extracted with hexane and
DCM. Both sub-extracts were then filtered and concentrated under vacuum (Laborata 4000 rotavapor) to yield dried residues of 2.6 % (DCM) and 2.4 % (hexane).

Extractions of Biological Material for NMR Analyses. Nectar extracts were obtained following the protocol recommended by Ralli et al. (2018) with minor modifications. Shortly, the nectars (150 mg) were suspended in deuterated solvent (D$_2$O, 1 mL) and the mixtures were stirred (sonication) for 10 min and then centrifuged at 10000 rpm for 15 min at 4°C. Supernatants were then recovered and transferred into 5 mm precision glass NMR tubes (New Era Enterprises, Inc.). Insect secretions were extracted from the glass pieces (previously fragmented) by immersion in methanol (room temperature, 1h). After methanol evaporation under reduce pressure, the residues were dissolved in D$_2$O and filtrated solutions were transferred into 5 mm precision glass NMR tubes. The D$_2$O-insoluble matter was used to produce a DCM extract that was analyzed by GCMS (see above).

Purification of Xanthoxylin from Plant Material. Xanthoxylin was isolated from a methanol (90% v/v) extract of aerial parts of *S. schottiana* (leaves and stems, 1 kg) previously dried and ground following a previous report (Yunes et al. 1990). The methanol maceration was performed with 2 L x 2 times, at room temperature for 5 days. The crude extract was further fractionated by an open column chromatography (mobile phase: hexane/DCM, 1:1) in which 40 fractions (15 mL) were collected. Xanthoxylin eluted from fractions 16 to 22 (Rf = 3.6). Visualization in TLC plates (mobile phase as in the column chromatography) was done by UV (254 nm) and anisaldehyde (Reagents Merck 1980). From half of the crude extract 54 mg of xanthoxylin were obtained (0.01 % in dried plant material).

Gas Chromatography Coupled to Mass Spectrometry (GCMS) Analyses. GCMS analyses on hexane and DCM extracts were carried out using a Shimadzu 2010 GC coupled to a Shimadzu QP2010 plus mass spectrometer (MS). Data were analyzed using the Shimadzu Corporation GC-MS Solution v2.50 software (1999–2006). Analyses were run on a AT-5 MS capillary column (Alltech, 30m × 0.25mm, 0.25µm). The carrier gas was helium at 1 mL/min. The oven temperature program was as follows: 40°C for 1 min, then an increase to 280°C at 7°C/min, and finally to 300°C at 15°C/min and maintained at 300°C for 4 min. Injector and detector temperatures were 250°C. Injections were performed in splitless mode, and the injection volume was 1 µL. MS parameters were: electron impact ionization at 70 eV ionization potential, m/z 27–400. Analyses were run acquiring by TIC and SIM (chosen ions were 181 y 196). For retention index calculations (Adams 2007), a mixture of n-alkanes (100 ppm each, in hexane) was injected in the splitless mode immediately after the samples. The solutions of all samples analyzed by GCMS were done in the corresponding solvent (DCM or hexane) at 2 mg/µL. In each set of analyses GCMS of only solvent were run as controls.

NMR Analyses. NMR spectra of nectars for untargeted metabolomics analyses were recorded on a Bruker AVANCE III 500 instrument equipped with a TXI probe (with z-gradient) operating at 500.13 MHz for 1H. For each sample, NMR spectra were acquired at 298 K using the zpg5 pulse sequence and accumulating 64 scans (acquisition time 2 s, D1 2s), with a spectral width of 14 ppm. Phase correction was performed manually; multipoint baseline was performed and apodization was exponential (0.3 Hz). $^1$H NMR chemical shifts were referenced to the solvent residual signal.

To characterize xanthoxylin NMR spectra of the isolated xanthoxylin and the standard (CDCl$_3$ solutions) were acquired on a Bruker AVANCE NEO 400 instrument equipped with a multinuclear BBFO Plus probe operating at 400.13 MHz for 1H and 100.62 MHz for 13C. NMR spectra were acquired at 295 K using the zg30 pulse sequence and accumulating 64 scans (acquisition time 4 s), with a spectral width of 14 ppm.
Data Processing. Chemical data from nectar samples were analyzed in bulk to obtain the matrixes that were then subjected to multivariate analyses [30, 31]. In the case of the GCMS analyses, raw data was processed using Paradise (Johnsen et al. 2017). After raw data processing with Paradise, matrices of 12 x 57 and 15 x 34 were obtained for DCM and hexane extracts, respectively. Once distinctive peaks were identified, metabolite annotation was based on comparison of their retention indexes with those reported by Adams (2007) and Pherobase database (El-Sayed 2012), and of their fragmentation patterns with those contained in the NIST 05 (Linstrom and Mallard 2005) and the SHIM 2205 (Adams 2007) libraries, as well as by comparison with a true standard obtained from Sigma-Aldrich (in the case of xanthoxylin). For NMR, raw data were processed using the licensed Mestre Nova 12.0 software (https://mestrelab.com/) in all cases. ¹H spectra were aligned, bucketed, and integrated using regular intervals of 0.01 ppm. The spectra were normalized to the total extracted mass of each sample, and the solvent signal regions were excluded (around 4.7 ppm) in the analyses. Three separate data matrices were obtained from the processed D₂O NMR spectra of the 36 samples (N = 18/ colony group): Matrix 1 (36 x 897) included de complete data set (all bins from 0 to 9.5 ppm), Matrix 2 (36 x 201) included signals from 3.5 to 5.5 ppm (corresponding to typical sugar signals), and finally Matrix 3 (36 x 696) included the spectral regions not included in Matrix 2 (0.7–3.5 ppm and 5.6–9.5 ppm, which represent the data without the typical signals from sugars).

Larval Assay. One-day-old larvae were collected from their cells, and reared at high humidity at 34°C in 96 plastic well microplates containing an excess of artificial diet (Evans 2004). Five groups of 24 larvae each were used (RD; XN_02; XN_04; control 1 and control 2), and the complete experiment was carried out by triplicate. All groups of larvae were fed with an artificial diet composed of 4/6 of royal jelly and 1/6 syrup (18% fructose, 18% glucose and 3% yeast extract). Besides that, larvae from RD group were fed with 1/6 nectar from affected colonies; larvae from XN_02 group were fed with 1/6 nectar from healthy colonies inoculated with a 0.2% of xanthoxylin; larvae from XN_04 group were fed with 1/6 nectar from healthy colonies inoculated with a 0.4% of xanthoxylin; larvae from control 1 were fed with 1/6 nectar from healthy colonies and finally larvae from control 2 were fed with 2/6 of syrup instead of 1/6. After the first 48 hours, larvae were taken out of the incubator and examined daily for five days. Every day, surviving larvae were transferred to new microplates filled with fresh artificial food, according to the different treatments.

The xanthoxylin concentration in diets (0.2% w/w) was calculated from the concentrations found in NMR analyses of nectars from RD-affected colonies. To estimate this amount, samples from nectars from RD-affected colonies (150 mg) were prepared as explained above to be analyzed by NMR and added with 1µL acetone (1.4E-5 mol). The comparison between the area of the six H (integrated as 1) in acetone and the area of the two aromatic H (integrated as 0.04) in xanthoxylin allowed this estimate.

Statistics. Multivariate data analyses of chemical data were performed using the Metaboanalyst platform (Xia and Wishart 2016). Chemical profiles were first explored with unbiased Principal Component Analyses (PCA). Further analyses for identifying peaks present only in RD nectars were done using Partial Least Square -Discriminant Analyses (PLS-DA). In these supervised models, the health status (with or without RD) of each colony was included in the model. The PLS models were cross validated with permutation tests (number of permutations as indicated in each result). Then, the PLS loading and variable influence on the projection (VIP) scores were used to make an initial selection of peaks of interest (VIP > 2) (Xia and Wishart 2016). Since our initial hypothesis was that the nectars from RD-affected colonies should have some substance in higher concentrations than nectars from healthy colonies, we focused on signals not only with high VIP but also that they were present in higher concentrations in nectars from RD-affected colonies.
For the larva bioassay, a survival analysis was performed with the Kaplan-Meier method. The difference between the survival curves was calculated with the Log-rank (Mantel-Cox) test using GraphPad Prism Version 8.4.0 for Mac, GraphPad Software, San Diego, California USA (www.graphpad.com).

Results And Discussion

The strategy to identify chemicals that may act as toxins in the RD-affected colonies included as a first step the untargeted search of metabolites present in nectar samples from those colonies but not present in nectars from healthy colonies (analyzed by NMR and GCMS analyses). Once different signals were identified in the RD nectars, xanthoxylin was isolated from plant material and targeted searches by GCMS in *E. cestri* secretions and *S. schottiana* aerial parts were done. Comparisons among these samples allowed us to detect xanthoxylin as a potential xenobiotic that might cause the larval mortality observed in RD-affected colonies. Finally, a larval feeding assay where larval diet was xanthoxylin enriched allow us to identify xanthoxylin as the potential toxic that causes RD.

NMR of Nectars. The multivariate analyses of the NMR data were performed on matrixes previously normalized by data scaling and data transformation (Table S2). The principal component analysis (PCA) on the different matrixes allowed exploratory analyses of the data. The PCA (PC1 63%, PC2 9%, PC3 5%) of the metabolic fingerprints (matrix 1, all bins considered) from nectar samples in D$_2$O showed that they can be differentiated according with the presence of RD (Fig. S1A). Since sugars account for most of the nectar composition, two other data sets were also analyzed: Matrix 2, with bins corresponding to the shift range of sugars, and Matrix 3, with all other bins. In both cases, results also showed differential clustering of nectars from RD and healthy colonies, suggesting a different composition among nectars. In the case of the matrix corresponding to the data from matrix 2 (bins from the "sugar region") the variance was accounted as follows: PCI, 34 %; PC2, 18% and PC3, 11% (Fig. S1B). For matrix 3 (all data but the "sugar region") the variance was well explained by 2 components (PC1 80%, PC2 5%; Fig. S1C). Models from Partial Least Squares - Discriminant Analysis (PLS-DA) were then built for the three matrices (Fig. S1D-F). In all cases, the models passed permutation test (R$^2$, Q$^2$ and p values are shown in Table S2). The differences between both nectars was expected as the origin of the nectar raw material impacts on nectar composition (Kortesniemi et al. 2016). Besides, the variability among samples from RD colonies was always lower than among samples from healthy colonies (Fig. S1), a fact that was also evidenced by the results of random forest analyses run on these data (Table 1). Random forest ability to predict the nectar type was always higher for RD samples than for healthy ones, probably correlating to a higher variability in nectars from healthy colonies (Kortesniemi et al. 2016; Schievano et al. 2012; Simova et al. 2012). Since healthy colony samples came from several different locations, with surely different floral offerings, these honeybees would be in contact with more diverse floral resources which could explain the higher variation observed. On the other hand, samples from RD-affected colonies came from fewer locations (see Supplementary material Table S1). These results also suggest the presence of differential compounds in nectars from colonies with and without RD. From the models built, 43 bins with a Variable Importance in Projection (VIP) greater than 2 were identified (Chong et al. 2019). Among these 43 bins, 24 corresponded to the "sugar region" (3.5–5.5 ppm), which would indicate a different sugar composition in nectars from colonies with and without RD (even though we cannot rule out the presence of other compounds with chemical shifts in this region). Indeed, a different sugar composition in honeys of different origins has widely been well documented (Kortesniemi et al. 2016; Schievano et al. 2012; Simova et al. 2012) and it is consistent with what is described in the FAO standards on chemical differences between honeydew and honeys (CX 12-1981 2019). Bins with high VIP in the PLSDA of matrix 3 (shifts from compounds other than sugars) were also detected.
Among these bins, the ones present in higher amounts in nectars from RD-affected colonies included chemical shifts corresponding to the aromatic region and around the 3-ppm region. Overall, these results indicate a differential composition of nectars from both kinds of colonies not only related to the saccharide composition but also to other kind of metabolites, allowing to distinguish between nectars from healthy and RD-affected colonies by \(^1\)H NMR untargeted metabolomics. However, the great complexity of the matrixes, and the fact that metabolites other than saccharides and aminoacids are in much lower amounts make the identification hard. For that reason, we focus our efforts on the extracts analyzed by GCMS.

Table 1
Random Forest Classification Performance for the Three Data set Analyzed
(calculation were done using the Metaboanalyst platform (Xia and Wishart 2016).

|                | Healthy | RD  | Classification error | Classification error (out of bag) |
|----------------|---------|-----|-----------------------|----------------------------------|
| **Matrix 1 (All bins)** |         |     |                       |                                  |
| Healthy        | 14      | 4   | 0.22                  | 0.139                            |
| RD             | 1       | 17  | 0.06                  |                                  |
| **Matrix 2 ("sugar region")** |         |     |                       |                                  |
| Healthy        | 16      | 2   | 0.11                  | 0.056                            |
| RD             | 0       | 18  | 0                     |                                  |
| **Matrix 3 ("no sugar")** |         |     |                       |                                  |
| Healthy        | 13      | 3   | 0.19                  | 0.118                            |
| RD             | 1       | 17  | 0.06                  |                                  |

GCMS of Nectar Extracts. The matrixes from the Paradise-processed GCMS data were subjected to principal component analysis (PCA) to visualize the differences between the healthy and RD nectars. Only in the case of the hexane extracts a clear separation of the chemical profiles of RD colonies compared to healthy ones could be observed (Fig. S2). Further, PLS-DA on data form DCM extracts did not validate (p = 0.86–860/1000). The PCA (singular value decomposition) on the identified peaks from hexane extracts showed that the data was well explained by 2 components (Component 1: 57.7%; Component 2: 25.9%, Fig. S2A) and the peaks that better explained the variance in the data (higher loadings) were at retention times of 17.2, 21.8 and 22.1 min. A partial least squares-discriminant analysis (PLS-DA, Fig. 1) was then used to model the differences between both kinds of samples. Permutation tests based on separation distance were applied to evaluate the reliability of the model (2000 permutations, p = 0.053, Fig. 1B). Overall, the PLS-DA model was found to be a good model for discrimination between both nectars. The validated model had one component, with \(R^2 = 0.69, Q^2 = 0.46\) and accuracy of 0.83). The 3 most important peaks identified by the VIP scores were at the same retention times already identified by the PCA (17.2, 21.8 and 22.1 min, Fig. 1C). These discriminating metabolites (Fig. 1D-F) were then tentatively identified at the level of compound class from mass spectra acquired during the GCMS analyses (at the identified retention time). The peaks at 17.2 (retention index 1410) and 21.8 (retention index 1650) were classified as sesquiterpenes by their mass spectra (Fig. S3). The compound eluting at 22.1 min had a characteristic mass spectrum [181 (100), 196 (31), 166 (12), 95 (11), 138 (10), 182 (9), 69 (9), 42 (8), 178 (7), 123 (6), 53 (5), 110 (5), 51 (4), 125 (3), 197 (3), 93 (3), 151 (3), 79 (3), 38 (3), 137 (3), 111 (3), 65 (3), 135 (2), 77 (2), 109 (2), 66 (2), 55
(2), 81 (2), 121 (2), 67 (2), 153 (2), 179 (2), 108 (2), 80 (2), 52 (2), 107 (2), Fig. 2A], that exhibited a 93% and 91% similarity with the mas spectra registered for xanthoxylin (1) in the Shim (Adams 2007) and NIST (Linstrom and Mallard 2005) Libraries respectively, and with the mass spectra of a standard of xanthoxylin (Fig. 2B). This spectrum was also like the one from the true standard (Fig. 2B). The retention index calculated for this compound was 1662, similar to the one reported in the Shim Library (1667). Quantification from GCMS data of the corresponding peak show a significant difference between the content in nectars from RD and healthy colonies (p = 0.02, t-test, Fig. 3).

S. schottiana have been reported as a good source of xanthoxylin (Calixto et al. 1990; Lima et al. 1995). Since the other detected metabolites (Fig. 1) were terpene compounds usually found in plants used by honeybees when foraging (Pham-Delegue et al. 1990; Zhang 2018), and also usually found in honeys (Pontes et al. 2007) we focused our efforts on xanthoxylin as the potential toxic substance accounting for the RD effects. To sustain such hypothesis, we explored whether xanthoxylin was in both, excretions from E. cestri feeding on S. schottania, and the plants themselves (S. schottiana).

Xanthoxylin Detection in Plant Material by TIC and in E. cestri by SIM. The GCMS profiles from total ion chromatograms (TIC) of extracts from plant material and E. cestri honeydew excretions were compared to the profiles from nectars collected from RD-affected and healthy colonies (Fig. 4). The xanthoxylin previously found in extracts from RD-affected colonies (Fig. 1–3) was also detected in plant material at 22.1 min (Fig. 4). When comparing the spectra of the peak at this retention time in the plant material extract, the same characteristic spectrum as in the case of samples from RD-affected colonies was found in both samples (Fig. 2C). However, in the case of the extract of E. cestri secretions, the amount of the sample precluded a conclusive matching. To confirm the presence of xanthoxylin in E. cestri honeydew excretions, the samples were analyzed by SIM GCMS choosing ions 181 and 196 (Fig. 2) to be monitored (Fig. 5). Such analyses allowed for the detection of a peak at the same retention time as xanthoxylin with m/z fragments 181 and 196 in E. cestri samples as well as nectars from RD-affected colonies, but not present in honeys form healthy colonies (Fig. 5).

Xanthoxylin Characterization. To confirm its chemical structure, the targeted compound was purified from the plant extracts (Calixto et al. 1990). Xanthoxylin (C_{10}H_{12}O_{4}, 2'-Hydroxy-4',6'-dimethoxyacetophenone, 1) was isolated as a crystalline white powder; \(^{1}H\) NMR (400 MHz, CDCl3, Fig. S4) \(\delta \) 14.06 (b, 1H); 6.08 (d, 1H, \( J = 2.4 \)); 5.94 (d, 1H, \( J = 2.4 \));3.87 (s, 3H), 3.84 (s, 3H); 2.63 (s, 3H). 13C NMR (101 MHz, CDCl3) \(\delta \) 203.17 (C1), 167.60 (C4'), 166.09 (C6'), 162.91 (C2'), 106.02 (C1'), 93.48 (C5'), 90.75 (C3'), 55.55 (broad peak for both OMe), 32.92 (C2).The recorded spectroscopic data agreed with the previously reported (Calixto et al. 1990) and also were compared to the one obtained from a true standard (Sigma-Aldrich, 630586-5G, Lot # MKBX3506V). The compound was then identified as 2'-Hydroxy-4',6'-dimethoxyacetophenone -xanthoxylin- (1). The MS spectrum was as described above.

Larva Bioassay. Once we had detected xanthohxylin in the different biological samples (plant material, E. cestri and nectars from RD-affected colonies), we performed a bioassay where larvae were fed on xanthoxylin-enriched diets to mimic the effects of RD. Artificial diet normally used for larval rearing was modified to include nectar from healthy or RD colonies, or nectar from healthy colonies supplemented with xanthoxylin. Finally, one group of larvae just received normal artificial diet.

Results indicated that the modification of the artificial diet to include nectar from healthy colonies did not affect larval survival (Log-Rank test: p > 0.05). On the other side, feeding larvae with nectar from RD colonies, or healthy nectar with xanthoxylin at 0.2 and 0.4 % (XN_02 and XN_04 respectively), significantly reduced their survival
compared with larvae fed on healthy nectar or the artificial diet (Log-Rank test: \( P < 0.0001 \) in all cases, Fig. 6). Larvae fed on XN_04 showed a similar survival curve than those fed on RD nectar (Log-Rank test: \( p > 0.05 \)). In our previous work, we observed that the mortality of larvae fed with nectars from RD-affected colonies and \( E. \) cestri secretion was similar or lower than in these cases (Invernizzi et al. 2018). A mortality of more than 60 % at day 4 was observed in the groups fede with xanthoxylin (XN_02 and XN_04) and with nectar from RD-affected colonies. Therefore, we postulate that xanthoxylin may be involved in larval absence within the colonies, one of the main symptoms of RD syndrome.

Final considerations. In a recent study, Invernizzi et al. (2018) reported clear evidence that the massive death of one-day-old larvae with the main symptom of the RD syndrome was associated with the collection by foragers of the secretions of \( E. \) cestri feeding on \( S. \) schottiana trees. However, in that report the compounds involved in larval poisoning remained unidentified. In the present study, we show the first results of the chemical analyses and profile comparisons of nectars from healthy and RD-affected colonies. \(^1\)H NMR spectra of nectars from RD-affected colonies show several different signals than the spectra of nectars from healthy colonies. Some of these signals are indeed due to differences in the composition in carbohydrates and aminoacids, and future elucidation will allow us to chemical characterized these nectars. By GCMS we were able to show that nectars from diseased colonies share xanthoxylin (a metabolite which is absent in nectars from healthy colonies) with the extracts from \( S. \) schottiana and the secretion of planthoppers feeding on \( S. \) schottiana. As mentioned, xanthoxylin had been already reported from \( S. \) schottiana trees (Calixto et al. 1990). Thus, this compound was identified as the probable causal agent of the larval death, which was confirmed in the tests carried out feeding larvae in the laboratory.

As honeybees have been observed foraging on \( E. \) cestri secretions, our results suggest a pathway where \( S. \) schottiana trees produce xanthoxylin that is ingested by \( E. \) cestri. Then, \( E. \) cestri, in the process of filtration of the ingested sap to concentrate aminoacids, produce xanthoxylin rich secretions upon which bees forage. In this way, bees bring xanthoxylin to the colony that is incorporated in the nectar fed to 1st instar larvae which, as it was shown in the bioassay, died because of its ingestion. Xanthoxylin, first isolated from \( Xanthoxylum alatum \) (Rutaceae), has also been reported in different species belonging to the families Euphorbidae, Piperaceae, Apiaceae, Rutaceae, etc. (Chermenskaya et al. 2012). In relation to other biological activities, xanthoxylin has been previously reported as an antagonist of contractions of smooth and cardiac muscles of vertebrate in vitro (Calixto et al. 1990; Cechinel et al. 1995), and also exhibits activity against some bacteria of the urinary tract (De Godoy et al. 1991) and some fungus (Lima et al. 1995; Pinheiro et al. 1999). Indeed the antifungal activity of xanthoxylin supports the popular use of plant extracts containing this compound (Lima et al. 1995). Besides, there are only a few reports regarding anti-insect effects of xantholxylin containing extracts: it has been identified as the major constituent of active fractions from extracts of \( Ungernia severtzovii \) (Amaryllidaceae) that exhibited anti-aphid activity (Chermenskaya et al. 2012); and extracts of \( Zanthoxylum bungeanum \) (Rutaceae) that had deterrent effects on oviposition and feeding of \( Sitotroga cerealella \) (Lepidoptera: Gelechiidae) (Ge and Weston 1995). Our report here shows that xanthoxylin is also toxic to honeybee larvae. Even though we were able to mimic the RD effects on 1st instar larvae, when feeding them with xanthoxylin-reach diets, we cannot rule out that other compounds from the diet could also contribute to the toxicity of nectars in RD-affected colonies. Indeed, other compounds (8-hydroxyquinoline derivatives) with insecticidal properties have been reported in other \( Sebastiania \) species (Lee et al. 2010).

Honeybees can avoid collecting nectars that contain toxic substances from the plants due to a learning process where the honeybees associate a particular nectar with a toxic effect (Wright et al. 2010). However, they do not avoid collecting \( E. \) cestri secretions with toxic substances from \( S. \) schottiana. This apparent contradiction may
arise from the fact that these toxic substances do not affect the adult honeybees, precluding them from establishing this association. Likewise, it is unlikely that adult honeybees, which obtain food from several plants, are capable to associate a carbohydrate source with a larval intoxication within the colony. Nor can it be ruled out that honeybees, a generalist species that have few gustatory receptors, do not detect the low concentrations of xanthoxylin that are present in *E. cestri* secretions (Robertson and Wanner 2006).

Future work should focus on how xanthoxylin affects the physiology, morphology, and development of the larvae, as well as whether xanthoxylin has also an effect on apoptosis as it was already found for the ingestion of RD honeydew (Viotti et al. 2021). Besides, it would be interesting to know whether xanthoxylin is stable or breaks down over time in the nectars, as it does in aqueous solution (Kwon et al. 2014), changing in this way its toxicity over time.

To our knowledge, this is the first report not only of a xenobiotic present in the secretions of a planthopper that can emulate the RD syndrome in 1st-instar honeybee larvae, but also, we here showed evidence of an interspecific flow of xanthoxylin among three trophic levels that has relevant consequences on the survival of the honeybee colonies.

**Declarations**

**Acknowledgements** - We would like to thank Andrés López and Guillermo Moyna for technical assistance in the NMR analyses.

**Funding** - Financial support was received from grants from INIA (Instituto Nacional de Investigación Agropecuaria, FPTA 2012-322) and CSIC (Comisión Sectorial de Investigación Científica, Universidad de la República, Uruguay. *Programa Grupos, CSIC-Grupos 2004*).

**Conflicts of interest** - The authors have no conflicts of interest to declare that are relevant to the content of this article.

**Availability of data and material** - All relevant material was included in the manuscript or the supplementary material.

**Code availability** - Not applicable.

**Authors' contributions** - All authors contribute to the different experiments: CR, LA and ARB conceived and performed chemical analyses; DA, KA and CR performed larval assay. CI, ES and KA conceived the presented idea. ES and CI performed field sampling. CR wrote the manuscript with input from DA, KA, ES and CI. CR and CI were responsible of the funding grants.

**Ethics approval** - Ethics approval was not required.

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Figures
Figure 1

PLS-DA on GCMS data from hexane extracts of nectars from colonies with different health status: Scores plot between the component 1 and 2 with the explained variances in brackets (A). The PLS-DA model was validated by permutation tests based on separation distance (insert B, p = 0.056 (56/1000). C: Important features identified by the model (The colored boxes on the right indicate the relative concentrations of the corresponding metabolite in RD-affected colonies and healthy ones). D-F: relative concentrations of compounds with higher Variable Importance in Projection (VIP) scores (plots of normalized data are shown for peaks at 17.2 min -D-, 22.1 min -E- and 21.8 min -F).
Figure 2

Mass spectra of the compound eluting at 22.1 min in GCMS of hexane extracts of nectars from colonies with RD (A), a true standard of xanthoxylin (B) and plant material (C).
Figure 3

Relative quantification for the xanthoxylin in nectars from RD-affected colonies (black bars) and from healthy colonies (open bars) (P = 0.02, 2-Sample t-Test, assuming equal Variances).
Figure 4

GCMS profiles (acquired using the TIC mode) of extracts from: S. schottiana plant material (A, red trace), E. cestri (B, brown), nectars from RD-affected colonies (C, red) and from healthy colonies (D, orange). Insert shows peak of xanthoxylin, at rT 22.1 min.
Figure 5

SIM-GCMS profiles (m/z 181 and 196) of extracts from nectar from healthy (A), RD-affected (B) colonies, and from E. cestri secretions (C). The arrows indicate where both 181 and 196 were detected (rT = 22.1 min) indicating the presence of xanthoxylin.
Figure 6

Survival curves of laboratory reared larvae fed with different diets. All larvae were fed with an artificial diet composed of 4/6 of royal jelly and 1/6 syrup. Besides that, larvae from RD group were fed with 1/6 nectar from affected colonies; larvae from XN_02 group were fed with 1/6 nectar from healthy colonies inoculated with a 0.2% of xanthoxylin; larvae from XN_04 group were fed with 1/6 nectar from healthy colonies inoculated with a 0.4% of xanthoxylin; larvae from control 1 were fed with 1/6 nectar from healthy colonies and finally larvae from control 2 were fed with 2/6 of syrup instead of 1/6.

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