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Host and gut microbiome modulate the antiparasitic activity of nectar metabolites in a bumblebee pollinator

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Abstract

Antimicrobial nectar secondary metabolites can support pollinator health by preventing or reducing parasite infections. To better understand the outcome of nectar metabolite-parasite interactions in pollinators, we determined whether the antiparasitic activity was altered through chemical modification by the host or resident microbiome during gut passage. We investigated this interaction with linden (*Tilia* spp.) and strawberry tree (*Arbutus unedo*) nectar compounds. Unedone from *A. unedo* nectar inhibited the common bumblebee gut parasite *Crithidia bombi in vitro* and in *Bombus terrestris* gynes. A compound in *Tilia* nectar, 1-[4-(1-hydroxy-1-methylethyl)-1,3-cyclohexadiene-1-carboxylate]-6-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranose (tiliaside) showed no inhibition *in vitro* at naturally occurring concentrations, but reduced *C. bombi* infections of *B. terrestris* workers. Independent of microbiome status, tiliaside was deglycosylated during gut passage, thereby increasing its antiparasitic activity in the hindgut, the site of *C. bombi* infections. Conversely, unedone was first glycosylated in the midgut without influence of the microbiome to unedone-8-*O*-*β*-D-glucoside, rendering it inactive against *C. bombi*, but subsequently deglycosylated by the microbiome in the hindgut, restoring its activity. We therefore show that conversion of nectar metabolites by either the host or the microbiome modulates antiparasitic activity of nectar metabolites.

Keywords
Bee health, disease, phytochemistry, host-parasite ecology, gut microbiota, Trypanosomatidae

Introduction

The health of wild pollinators is under threat from parasites through a variety of anthropogenic factors, including the potential introduction of parasites into new geographic areas by global trade (Cameron et al. 2011, Schmid-Hempel et al. 2014), spill-over of emerging infectious diseases from managed pollinators like honeybees (Fürst et al. 2014; Wilfert et al. 2016), or through additive or synergistic effects between parasites and other manmade stressors like pesticides (Goulson et al. 2015; Siviter at al. 2021). Dietary secondary plant compounds naturally occurring in nectar or pollen could ameliorate these threats to pollinator health via increased tolerance, prevention, or reduction of infections (Richardson et al. 2015; Koch et al. 2017; Koch et al. 2019; Bernklau et al. 2019; Folly et al. 2021). Understanding the role of different foraging plants for pollinator diseases may thus present a promising avenue to promote pollinator health, for example by protecting natural habitats with key plant species (Koch et al. 2019) or promoting forage plants with health benefits through seed mixes in agricultural environments (Folly et al. 2021). However, we still lack a detailed understanding of the factors that determine the effects of dietary phytochemicals on parasites of pollinators within the host. Our ability to predict the outcomes of the diversity of possible phytochemical-pollinator-parasite interactions in the wild is therefore limited (Sutherland et al. 2020). Indeed, the effect of secondary nectar metabolites on parasites in pollinator hosts has, in some cases, been inconsistent or contradictory between studies in the same host-parasite system (e.g., Richardson et al. 2015; Palmer-Young et al. 2017a) and may be affected by host genotypes or environmental conditions like temperature and food composition (Thorburn et al. 2015; Palmer-Young et al. 2017a). *In vitro* screens of nectar and pollen

phytochemicals can provide insights into direct effects on pollinator parasites in culture, for example showing synergistic effects between compounds (Palmer-Young et al. 2017b), variation in resistance against compounds between different parasite genotypes (Palmer-Young et al. 2016), or effects on parasite cell morphology (Koch et al. 2019). However, without studying the fate of dietary phytochemicals in the host, we cannot establish whether these simplified *in vitro* experiments reflect conditions experienced by parasites *in vivo*, and thus whether they are ultimately relevant in ecological contexts (Koch et al. 2019).

The fate of phytochemicals after ingestion by pollinators before reaching parasite infection sites likely influences their antiparasitic effect. Plant compounds may be chemically transformed during passage through the bee gut (Vidkjær et al. 2021). This could either increase or decrease their activity against parasites. Koch et al. (2019), for example, found that callunene from heather nectar can reduce the likelihood of infections with the common trypanosomatid gut parasite *Crithidia bombi* in bumblebees (*Bombus terrestris*) when parasite cells were exposed for a short time in the crop. However, callunene concentration sharply declined during gut passage, and did not reach the site of infection in the hindgut. Consequently, existing infections remained unaffected by callunene ingestion. Although dietary phyotchemicals may not reach internal parasite infections in their ingested form, modification of these compounds post-ingestion could modulate their subsequent impact on parasites. However, we currently lack an understanding of the processes underlying these changes to phytochemical structures and concentrations.

Chemical modification of dietary secondary metabolites in the bee gut can be caused by host enzymes secreted into the gut (Berenbaum & Johnson 2015). Bees produce a range of enzymes that can metabolise dietary secondary metabolites, including cytochrome P450 monooxygenases (P450s) and glutathione transferases (GSTs) (Berenbaum & Johnson 2015; du Rand et al. 2015). However, detoxification gene diversity is reduced in honeybees and bumblebees compared to other insects (Sadd et al. 2015; Berenbaum & Johnson 2015). The resident gut microbiome of social bees (see Kwong et al. 2017) may therefore play an important additional role in metabolising dietary secondary compounds. Kešnerová et al. (2017), for example, showed that the microbiome metabolised flavonoid glycosides in the honeybee gut, but the extent and functional relevance of metabolic transformation of secondary metabolites by the bee gut microbiome is not well understood. The presence and composition of the bacterial microbiome in bumblebees has previously been shown to influence parasite infections with the gut parasite *C. bombi* (Koch & Schmid-Hempel 2011 & 2012; Mockler et al. 2018). The mechanisms for this health benefit remain unclear but could include changes to the chemical environment in the bee gut.

Here, we studied this interaction between the pollinator host, nectar phytochemicals, parasites, and the microbiome. Following the discovery of *in vitro* activity of monofloral honey extracts from linden (*Tilia* spp.) and strawberry tree (*Arbutus unedo*) against *C. bombi* in Koch et al. (2019), we investigated three key issues. First, we tested the antiparasitic activity of secondary metabolites from the nectar of these tree species through *in vitro* and *in vivo* experiments with the important European bumblebee pollinator species *B. terrestris*. Second, we investigated if chemical transformation of nectar secondary metabolites post ingestion modulated their antiparasitic activity. Third, we tested the role of the host and the gut microbiome in the transformation of ingested nectar secondary metabolites.

Methods

Nectar analysis

Nectar samples were collected from *Arbutus unedo* L. and *Tilia tomentosa* Moench trees growing at the Royal Botanic Gardens (RBG), Kew (Richmond, Surrey, UK) in October 2019 (*Arbutus*) and July 2015 (*T. tomentosa*). Flowers were gauze bagged to prevent removal of nectar by bees, and nectar was collected after one day with 10 µl glass capillaries (Drummond Scientific, Broomall, USA). *B. terrestris* gynes (i.e., potential queens) foraging on *A. unedo* at RBG Kew were caught in October 2019, and nectar filled crops dissected out for chemical analysis of contents. All samples were weighed (Mettler Toledo Balance XS105), extracted in 80% methanol (including macerating bumblebee crop samples with plastic pestles), briefly vortexed, held in the dark for 24 hours at room temperature, briefly vortexed again, centrifuged for 2 minutes at 3000 rpm, and supernatants stored at -20°C until further analysis (see also methods in Koch et al. 2019). Extracts were analysed via HPLC-MS (Velos-Pro, Thermo Fisher Scientific; with a photodiode array (PDA) and High-Resolution-Electrospray-Ionization-Mass-Spectrometry (HR-ESI-MS) on a Thermo Fisher Scientific LTQ Orbitrap with 5 µl injection volume on a Phenomenex Luna C18 (2) column (150 x 3 mm, 3

µm particle size) held at 30°C and a linear mobile phase gradient of 10%–100% aqueous MeOH containing 0.1% formic acid over 20 min. We focused our analyses on a major secondary metabolite from *Tilia* honey 1-[4-(1-hydroxy-1-methylethyl)-1,3-cyclohexadiene-1-carboxylate]-6-*O*-*β*-D-glucopyranosyl-*β*-D-glucopyranose (Naef et al. 2004; Frérot et al. 2006) (to which we assign the trivial name tiliaside) and a major component of *A. unedo* honey: the isoprenoid unedone (2-(1,2-dihydroxypropyl)-4,4,8-trimethyl-1-oxaspiro[2.5]oct-7-en-6-one) (Tuberoso et al. 2010). We quantified unedone and tiliaside with peak areas recorded at their UV absorbance maxima (unedone: 245 nm; tiliaside: 308 nm) and compared with calibration curves from pure standards between 1 ppm and 1000 ppm (see supplementary data).

Compound isolation and identification

Nectar compounds were isolated from monofloral honey of *A. unedo* (source: *Wild about Honey*, Portugal) or *Tilia* sp. (source: *Tesco*, UK; honey origin: Romania) respectively, as monofloral honey offers a source for bulk quantities of nectar compounds that are often similar to the chemical composition of the nectar from which it is derived (Koch et al. 2019).

To isolate unedone, *A. unedo* honey was dissolved in ultra-pure water (Milli-Q; Sigma, St. Louis, MO) in a 1:2 (weight/weight) ratio. The diluted honey was then mixed with ethyl acetate in a 1:1 ratio (volume/volume). The mixture was shaken in a separating funnel until an emulsion was formed and left to separate overnight. The ethyl acetate layer was removed and dried on a rotary evaporator. Extracts were re-dissolved in 80% methanol and partitioned on a flash chromatography system (Biotage Isolera One; Biotage, Sweden) using a SNAP Ultra C18 cartridge (water-methanol gradient: 5% methanol for 1.5 column volumes (CVs); 14% methanol: 1.5 CVs; 31% methanol: 5.5 CVs, 100% methanol: 1 CV). Unedone eluted at 5 CVs, and collection was guided by monitoring UV absorbance at 245 nm. Solvent was removed on a rotary evaporator, purity evaluated by ^{1}H NMR comparing to chemical shifts in Tuberoso et al. (2010) and extract stored at -20°C until further use.

A previously undescribed glycosylated derivate of unedone, unedone-8-*O*-*β*-D-glucoside, was isolated from *B. terrestris* gynes' faeces. For this, gynes collected at RBG Kew were housed in individual plastic boxes and fed with 50% Apiinvert sugar syrup containing 3.79 mM (910 ppm) unedone. Faecal material was harvested daily, either by collecting faeces with a 10 µl glass capillary from gynes periodically placed in plastic tubes, or by rinsing filter paper placed into the bottom of cages with ethanol. Faecal material was combined, filtered, dried down, and unedone-8-*O*-*β*-D-glucoside purified. First, faecal material was dissolved in H_2O, combined with an equal volume of ethyl acetate, shaken up in a separation funnel to an emulsion, and the ethyl acetate layer collected after the emulsion had separated. The ethyl acetate was dried down, resuspended in methanol and unedone-8-*O*-*β*-D-glucoside isolated on a flash chromatography system (Biotage Isolera One; Biotage, Sweden) using a SNAP Ultra C18 cartridge (water-methanol gradient: 5-30% methanol linear gradient: 7 CVs; 30% methanol: 4 CVs). Unedone-8-*O*-*β*-D-glucoside eluted at 8.9 CVs. To elucidate the structure, unedone-8-O-*β*-*D*-glucoside was dissolved in $CDCl_3$ and analysed by nuclear magnetic resonance (NMR) spectroscopy (400 MHz Bruker Avance; Bruker, Billerica, MA) using 1D (^{1}H, ^{13}C and DEPT) and 2D (^{1}H-^{1}H-COSY, ^{1}H-^{1}H ROESY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC) NMR spectroscopic analysis.

For extraction of tiliaside, *Tilia* honey was extracted in ethanol by mixing 1 part honey with 2 parts ethanol (by weight) in a conical flask, stirring the mix with a glass rod for 5 minutes and placing the mix on an orbital shaker for 1 hour, with additional stirring every 10 minutes. The ethanol was collected (leaving most of the sugar behind) and dried on a rotary evaporator until all the solvent had been removed. The extract was partitioned via flash chromatography (see above, but with the following water-methanol gradient: 10%-15% methanol: 5 CVs, 30% methanol: 3 CVs, 45% methanol: 2.5 CVs, 75%-100% methanol: 1.5 CVs). Tiliaside eluted at around 6 CVs, and the corresponding aglycone at around 8.5 CVs; collection was guided by monitoring UV absorbance at 308 nm. Tiliaside and the corresponding aglycone were further purified by semi-preparative HPLC on a Waters (UK) LC system (600E pump, 996 PDA detector; Phenomenex Luna C18 column: 150 mm x 10 mm, 10 µm particle size), and purity verified by ^{1}H NMR comparing to chemical shifts in Frérot et al. (2006). The structures for tiliaside and the aglycone of tiliaside were determined *ab initio* using NMR and MS data, and by comparison to the reported data by Frérot et al. (2006) (see supplementary information).

***In vivo* experiments**

For unedone *in vivo* experiments, *Bombus terrestris* gynes were collected in autumn 2018 at the Royal Botanic Gardens, Kew (Richmond, Surrey, UK). We selected gynes for the experiment, as it is the dominant

caste foraging on *A. unedo* at the time of flowering (personal observation; Stelzer et al. 2010). Individual gynes were housed in plastic boxes and fed with 50% Apiinvert sugar syrup (Apiinvert, Südzucker, Germany) and honeybee collected polyfloral pollen (Biobest, Belgium). Faeces of gynes was screened microscopically for parasite infections (*Crithidia*, *Nosema*, *Apicystis*) on the day of capture and after 2 weeks in the laboratory, and infected gynes were excluded. Uninfected gynes were randomly assigned to two treatments: a control treatment receiving 50% Apiinvert sugar syrup, and a unedone treatment receiving 50% Apiinvert sugar syrup containing 3.79 mM (910 ppm) unedone (around the limit of solubility of unedone in the diet, and below the average concentration measured in *B. terrestris* gyne crops foraging on *Arbutus* at RBG Kew). Both groups also received polyfloral pollen *ad libitum* (Biobest, Belgium). For *C. bombi* inoculations, gynes were deprived of food in individual plastic vials, and after 6 hours fed 15 µl of an inoculum containing 15,000 cells of *C. bombi* from a laboratory *in vitro* culture (source see strain details in Koch et al. (2019)) mixed 1:2 with 50% Apiinvert sugar syrup. After feeding on the inoculum, gynes were put back into their cages and received either the control or unedone diet for 7 days. As prevention of infection in gynes before hibernation can be expected to have major fitness benefits (Brown et al. 2003), we designed this experiment to test if feeding on unedone can reduce the risk of infections to queens. After 7 days, faeces was sampled from gynes and *C. bombi* cell concentrations determined with a Neubauer improved haemocytometer and phase contrast microscope (Zeiss Photomicroscope III; Carl Zeiss AG, Germany) at 640x magnification. We used an ANOVA with log-transformed *C. bombi* faecal concentrations as dependent and diet treatment groups as independent variable in R (R Core Team 2021). To test for effects of diet treatment on infection success, we used a χ^2-test in R (R Core Team 2021) scoring gynes without *C. bombi* in the faeces as uninfected (0) and gynes with any concentration of *C. bombi* as infected (1).
For the tiliaside *in vivo* experiment, *B. terrestris* workers were removed from colonies originating from wild-caught queens at RBG Kew, housed in individual plastic boxes with 50% Apiinvert sugar syrup and *ad libitum* polyfloral pollen (Biobest, Belgium) and infected with a laboratory strain of *Crithidia bombi* (see above, inoculum of 15,000 cells). After 7 days, infections were verified by microscopic examination of faeces, and uninfected individuals excluded from the experiment. Workers were then randomly assigned to feed on either a 50% Apiinvert sugar syrup control, or 9.88 mM (5000 ppm) of tiliaside in 50% Apiinvert sugar syrup. As many workers can be expected to be infected with *C. bombi* during the flowering period of Tilia in summer, but will have energetic costs from infections (Brown et al. 2000), we tested in this experiment if the feeding on tiliaside can reduce existing parasite loads. After 7 days, *C. bombi* infection levels were quantified microscopically from faecal samples, and log-transformed faecal *C. bombi* concentrations analysed via a linear mixed-effects model (lme) with treatment as fixed effect and colony as random effect with the function lme of the package nlme in R (Pinheiro et al. 2021). Gut fragments (crop, midgut, hindgut) were dissected from the bumblebees feeding on tiliaside in this experiment and analysed together with faecal samples for the degree of conversion of tiliaside to the corresponding aglycone of tiliaside (for analytical procedure, see microbiome experiment section). We tested for significant differences between proportions of the aglycone of tiliaside to tiliaside in the different gut segments with an ANOVA with logit-transformed proportions and Tukey's HSD test for pairwise comparisons in R (R Core Team 2021).

In vitro experiments

In vitro testing of all compounds was conducted following methods in Koch et al. (2019). Briefly, a *C. bombi* strain isolated from *B. terrestris* (details see Koch et al. 2019) was grown in standard *Crithidia* liquid medium (Salathé et al. 2012) at 28°C and 3% CO_2. Inhibition of *C. bombi* growth was tested in 96 well tissue culture plates (Eppendorf, Germany) with compounds dissolved in the culture medium in a dilution series with 2-fold concentration changes per step (6.25 mmol/l to 0.78 mmol/l for unedone/unedone-8-*O*-*β*-*D*-glucoside; 20 mmol/l to 2.5 mmol/l for tiliaside & the corresponding aglycone). To facilitate solubilizing compounds, we first dissolved compounds in methanol, and added dissolved compounds to the culture medium to make up a final concentration of 1% methanol. Choice of concentration ranges reflected concentrations found in nectar but had to be limited to a maximum of 6.25 mmol/l for unedone (1500 ppm), below the maximum natural concentration in nectar found in our study, but at the limit of solubility in the culture medium. The test medium with 1% methanol was included as negative control. An aliquot of 20 µl of a 1000 cells/µl *C. bombi* culture was mixed with 180 µl of test medium in each cell. After incubation at 28°C and 3% CO_2 for 7 days, *C. bombi* cell concentrations were determined microscopically with a Neubauer improved haemocytometer and phase contrast microscope (Zeiss Photomicroscope III; Carl Zeiss AG, Germany) at 640x magnification. Dose-response curves and estimates of the effective dose 50 (ED50) were

calculated with the drm function of the package drc in R (Ritz et al. 2015), using a 3-parameter log-logit model (fct = LL.3).

Microbiome experiment

Previous experiments by Koch & Schmid-Hempel (2011) suggest that newly emerged bumblebees lack the core resident microbiome and acquire it through social contact in the colony post emergence. To create microbiome depleted or colonised bumblebees, we here follow procedures outlined in Koch & Schmid-Hempel (2011) but expand on their procedures by surface sterilising pupae and maintaining newly emerged bumblebees in sterile, air filtered environments to prevent subsequent environmental contamination: *B. terrestris* worker and male pupae were carefully removed from cocoons of laboratory colonies (Biobest). Cocoons were opened and pupae removed with superfine stainless-steel forceps (flame sterilised between individuals) from cocoons at a stage of development with the cuticula mostly or completely dark (corresponding to pupal stages P14-P16 in Tian & Hines 2018). Pupae were then transferred to a laminar flow hood, immersed in sterile PBS with bleach (0.2% calcium hypochlorite, freshly prepared on the day) for 1 minute for surface sterilisation, rinsed in autoclaved PBS twice to wash off bleach, and placed onto autoclaved filter paper to remove the PBS. Bumblebee pupae were then incubated in sterile polypropylene containers with a cover containing a filter strip allowing for sterile gas exchange (OS140BOX, round model, 140 mm height, 90 mm diameter; Duchefa Biochemie, Haarlem, NL) at 30°C & 80% humidity. During all stages of the experiment, containers were only opened under a laminar flow hood to prevent microbial contamination, and all handling of bumblebees or contents of the container was conducted with sterilised implements. Once every morning, containers were checked for emergence of individuals, bumblebees removed, kept for 3 hours in sterile 90 mm petri dishes, and fed either 15 µl sterile 50% Apiinvert sugar syrup (control) or 15 µl of a 2:1 mix of 50% Apiinvert sugar syrup mixed with freshly collected faeces from 5 workers within the mother colony (to transplant the gut microbiome, see Koch & Schmid-Hempel 2011). Individuals that fed on the inoculum within 1 hour were then placed back into their container and kept at 26°C. Sterile pollen was provided to all individuals from polyfloral honeybee collected pollen (Biobest, Belgium) that was ground to a powder and soaked in 70% ethanol for 1 hour. Pollen was then spread out in a thin layer in sterile glass dishes in a laminar flow hood, and air dried for 24 hours to remove the solvent. Aliquots of approx 0.5 g sterilised pollen were placed into small containers made from 1.5 ml Eppendorf tubes cut in half and using the inverted top half with the lid closed. Pollen diet aliquots were stored at -20°C until use. All individuals were first fed on filter sterilised 50% Apiinvert sugar syrup and pollen for 7 days to allow for establishment of the microbiome and then split to either receive 3.79 mmol/l (910 ppm) unedone or 9.88 mmol/l (5000 ppm) tiliaside in 50% sugar syrup for a further 2 days. Diets were sterile filtered (Stericup sterile vacuum filtration system; Millipore, Burlington, USA) and 5 ml of sterile diet was presented to each bumblebee in 7 ml inverted Sterilin polystyrene containers (Sterilin Ltd, UK) with small holes over the rim of the lid for access. After 2 days on the unedone or tiliaside diet, bumblebees were removed from containers, chilled on ice, decapitated, dissected under a laminar flow hood with sterilised implements, and gut fragments (crop, midgut, hindgut) placed individually into sterile, weighed 1.5 ml Eppendorf tubes. Gut weights were determined on a Mettler Toledo Balance XS105 scale.

Gut fragments were macerated with sterile plastic pestles in 30 µl sterile 1/8 strength Ringer's solution and 5 µl transferred into a separate 1.5 ml Eppendorf tube for culturing. The 5 µl gut macerates were serially diluted by a factor of 10 for 3 times in sterile 1/8 strength Ringer's solution, and 5 µl of each dilution step plated out on Brain Heart Infusion agar plates. Plates were incubated for 5 days at 35°C and 5% CO_2 and colony forming units counted. We note that some bacterial members of the bumblebee microbiome are fastidious and would show poor or no growth under our culturing conditions, and consequently the absence of microbial growth on the culture plates does not necessarily indicate microbial sterility. A 50 µl ethanol aliquot was mixed with the remaining original 25 µl macerated gut for metabolite extraction, sonicated for 5 minutes and left for 24 h at room temperature. Suspensions were then spun down (3000 ppm, 3 min) and supernatants collected into autosampler vials for HPLC-MS analysis. Samples were analysed by High-Resolution-Electrospray-Ionization-Mass-Spectrometry (HR-ESI-MS) on a Thermo Fisher Scientific LTQ Orbitrap with photodiode array (PDA) detector. Target compound peaks were verified by mass of the pseudomolecular ions in positive mode and by comparison to standards of pure compounds. UV absorbance of the glycosides and corresponding aglycones (308 nm for tiliaside and aglycone of tiliaside; 245 nm for unedone and unedone-8-*O*-*β*-D-glucoside) were measured to estimate ratios of glycosides to aglycones in the different gut segments.

Results

Presence of unedone & tiliaside in nectar

Unedone, previously characterised from *Arbutus* honey (Tuberoso et al. 2010), was found in the HPLC-MS analysis of strawberry tree (*A. unedo*) nectar, verified with a unedone standard isolated from *Arbutus* honey (see methods, NMR data see Fig. S8), showing a matching *m/z* 241 pseudomolecular ion $[M+H]^+$ in positive mode at retention time 9.35 mins with a UV absorbance maximum of 245 nm. The accurate mass of the *m/z* 241 pseudomolecular ion $[M+H]^+$ in positive mode analysed via HR-ESI-MS furthermore matched the predicted mass from the molecular formula of the $[M+H]^+$ ion (observed m/z 241.1435; Δ ppm 0.350 versus expected for $C_{13}H_{21}O_4$) and had a matching MS2 spectrum (see Figure S10). Quantification using peak areas of UV absorbance gave a nectar concentration at an average of 14.66 mmol/l (3518 ppm) unedone (n = 7; range: 6.34 mmol/l - 35.8 mmol/l). Crop contents of *B. terrestris* gynes foraging on *Arbutus unedo* had an average of 9.15 mmol/l (2195 ppm) unedone (n = 3; range 7.48 mmol/l - 11.42 mmol/l).

Tilia tomentosa nectar contained tiliaside (verified by comparison to a pure standard isolated from *Tilia* honey (see methods, NMR data see Table S2 & Fig. S11, see also Frérot et al. 2006) and accurate mass of the main $[M+NH_4]^+$ (m/z = 524.2336; Δ ppm -0.356 versus expected for $C_{22}H_{38}O_{13}N$) and $[M+H]^+$ (m/z = 507.2072; Δ ppm 0.044 versus expected for $C_{22}H_{35}O_{13}$) pseudomolecular ions in positive mode analysed via HR-ESI-MS), as well as the matching MS2 spectrum (Figure S13). Quantification using UV absorbance (308 nm) peak area gave an average concentration of 16.74 mmol/l (8469 ppm; n = 3). This is similar to the 11.86 mmol/l (6000 ppm = 0.6%) of tiliaside reported by Frérot et al. (2006) in Swiss linden honey (likely from *Tilia cordata* or *Tilia platyphyllos*).

***In vivo* effects of unedone and tiliaside, and conversion during gut passage**

B. terrestris gynes feeding on 3.79 mmol/l (910 pm) unedone from *A. unedo* had lower infection levels in faeces samples 7 days after inoculation compared to the sugar water fed control group (Fig. 1A; ANOVA: F(1, 55) = 15.02, p = 0.00029), and were less likely to have developed an infection (χ^2 = 10.63, p = 0.0011; 96% infected in control group vs 60% infected in unedone group; n = 57). Tiliaside from *Tilia* nectar reduced *C. bombi* infection levels in faeces of *B. terrestris* workers with pre-established *C. bombi* infections after feeding on 9.88 mmol/l (5000 ppm) tiliaside for 7 days, compared to the sugar water fed control group (Fig. 1B; lme: F(1, 26) = 9.8, p = 0.0042).

HR-ESI-MS analyses showed that tiliaside was partially deglycosylated during gut passage in *B. terrestris* workers. The proportion of the corresponding aglycone 4-(1-hydroxy-1-methylethyl)-1,3-cyclohexadiene-1-carboxylic acid (observed $[M+H]^+$ m/z = 183.1016, Δ ppm 0.050 versus expected for $C_{10}H_{15}O_3$; MS2 spectrum see Figure S14, NMR data see Table S2, Figure S12) to tiliaside was low in the crop (average 9%; n = 15) and midgut (5%; n = 15), but significantly increased in hindgut (18%; n = 15) and faeces (27%; n = 16) (Tukey's HSD test, see Fig. S1).

A peak with similar UV absorbance to unedone (peak 245 nm) but higher mass (observed $[H+M]^+$ m/z = 403.1960, Δ ppm -0.617 versus expected for $C_{19}H_{31}O_9$; MS2 spectrum see Figure S9), consistent with a hexoside of unedone was recorded in an extract of faeces from bumblebee gynes fed on diets containing unedone. NMR spectroscopy of the compound purified from faeces of *B. terrrestris* gynes fed on unedone showed it to be a previously undescribed compound, unedone-8-*O*-*β*-D-glucoside (Fig. 2A, Figs. S2-S7, Table S1). The structure of the compound was determined as a mono-glycosylated derivative of the known unedone (Tuberoso et al., 2010), using 1D (^{1}H, ^{13}C and DEPT), 2D (^{1}H-^{1}H-COSY, ^{1}H-^{1}H ROESY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC) NMR spectroscopic analysis and MS. The ^{13}C NMR data for unedone (Tuberoso et al., 2010), shown in Table S1 (supplementary information as 1a) were comparable to those of the aglycone of this compound, except for C-8 which was deshielded at δ_C 82.3 (unedone gives δ_C 72.1). The analysis of the HMBC (see Table S1) showed that the C-8 position was substituted with a glycosidic moiety, and comparison of the reported data for β-glucose (Langat et al., 2021) (Table S1), and those of this compound were similar, hence the sugar unit was tentatively assigned as β-glucose. Use of a model and ^{1}H-^{1}H ROESY allowed for the assignment of the relative configuration of the compound, with an epoxy group on one side and the hydroxy group and glucose groups on the other face of the molecule, as shown in Figure S3, and the compound determined as unedone-8-*O*-*β*-D-glucoside.

***In vitro* activity of unedone vs unedone-8-*O*-*β*-D-glucoside & tiliaside vs aglycone of tiliaside**

Unedone inhibited *C. bombi* at 3.125 mmol/l and 6.25 mmol/l (considerably below the average of 14.66 mmol/l measured in nectar) (Fig. 2A). We estimated the effective dose 50 (ED50) for unedone at 3.94 mmol/l. In contrast, unedone-8-*O*-*β*-D-glucoside showed no inhibition of *C. bombi in vitro* up to a

concentration of 6.25 mmol/l, the highest concentration that was feasible for us to measure. This suggests glycosylation of unedone in the bumblebee gut will remove its antiparasitic activity. Tiliaside from linden nectar had no activity against *C. bombi* up to 20 mmol/l (higher than the concentration found in *Tilia* nectar by us), while the corresponding aglycone of tiliaside reduced *C. bombi* growth at concentrations from 5 mmol/l to 20 mmol/l with an estimated ED50 of 6.35 mmol/l (Fig. 2B). Conversion of the ~17 mmol/l tiliaside in nectar into the corresponding aglycone HMDA would therefore lead to strong inhibition of *C. bombi*.

Microbiome experiments

Microbiome inoculated bumblebee workers had an average of 1.9 X 10^6 colony forming units (CFUs), and males 5.9 X 10^6 CFUs per hindgut on BHI agar, with all individuals showing microbial growth. Hindguts of microbiome depleted males and workers had no microbial growth on BHI agar, even when plated out at the lowest dilution step (1/60th of total hindgut plated out), except for a single individual each of the males and workers having one CFU at the lowest dilution step (possible contaminant). This suggests that our experimental protocol was effective for reducing microbial colonisation of the gut and restoring it in the microbiome inoculated individuals.

Only unedone, but not its glucoside, was detected in the crop of both workers and males, independent of microbiome status (Fig. 3A). In contrast, in both workers and males, the unedone-8-*O*-*β*-D-glucoside was highly dominant (>90%) over unedone in the midgut, suggesting that unedone is glycosylated in this gut compartment (Fig. 3A). No difference was apparent between microbiome depleted and microbiome inoculated individuals, suggesting that the glycosylation likely derives from enzymes of the bumblebee secreted into the midgut. A reversion back to a higher proportion of unedone to unedone-8-*O*-*β*-D-glucoside was recorded in the hindgut of microbiome inoculated males and workers, suggesting partial deglycosylation in this gut compartment. However, the reversion to unedone was not apparent in microbiome depleted males and limited in microbiome depleted workers (Fig. 3A), suggesting that the deglycosylation of unedone-8-*O*-*β*-D-glucoside was caused by the hindgut microbiome. A schematic representation of the fate of unedone in the gut is given in Fig. 3B.

The proportion of tiliaside to its aglycone increased from the crop to the midgut and hindgut (Fig. 3C). Neither workers nor males showed apparent differences in the proportion of tiliaside to its aglycone between microbiome inoculated and microbiome depleted individuals (Fig. 3C), implying that the microbiome did not play a major role in this conversion, but that it is likely host induced.

Discussion

We demonstrate that the conversion of nectar secondary metabolites in the gut of bumblebees can modulate their activity against the common gut parasite *C. bombi*. Importantly, the activity of secondary compounds can both be increased or decreased during gut passage, showing that a better understanding of the fate of nectar or pollen secondary metabolites after ingestion is necessary to determine their effects on parasites of pollinators. Simplified studies of secondary metabolites from pollen or nectar in *in vitro* assays alone may therefore not accurately predict their interactions and effects on parasites in the host, as they may either over- or underestimate effects. Our findings furthermore show that the anti-parasitic activity of dietary secondary metabolites in a host can be altered by chemical changes induced by either the host or the resident microbiome. The effect of anti-parasitic secondary metabolites on host infections will therefore likely depend on factors like the host genetic background (e.g., regarding enzymes processing secondary compounds), differences in host gene expression of enzymes metabolising secondary compounds, gut pH (Poreddy et al. 2015), and the composition and activity of the microbiome.

Differences in activity against *C. bombi* were driven by changes in glycosylation: for both *Arbutus* and *Tilia* nectar metabolites, aglycones had higher activity than corresponding glycosylated compounds. Similarly, Tasdemir et al. (2006) found generally higher *in vitro* activity of flavonoid aglycones against several human pathogenic trypanosomatid species than corresponding glycosides. Glycosylation increases polarity and therefore water solubility of secondary metabolites but decreases their ability to cross cell membranes (Vasudevan & Lee 2020). Therefore, a possible explanation for the higher antiparasitic activity of the two aglycones studied here is their increased ability to cross *C. bombi* cell membranes relative to their corresponding glycosides. Once in the cytosol of the parasite, the secondary compounds will be able to interfere with cellular processes of the parasite, although exact mechanisms and cellular targets were not

studied here. Our findings suggest that antiparasitic effects of glycosylated nectar or pollen metabolites will be underestimated if they can undergo deglycosylation in the host. Tiliaside from linden tree nectar was deglycosylated to the same extent in microbiome depleted or microbiome inoculated bumblebees, suggesting that this transformation was dependent on the host, not the microbiome. Bees produce *β*-glucosidases to catalyse the cleavage of *β*-glycosidic bonds in the crop and midgut (Pontoh & Low 2002), which are likely responsible in our experiment for the deglycosylation. In the case of unedone-8-*O*-*β*-D-glucoside, deglycosidation in the hindgut was increased in microbiome inoculated bumblebees, suggesting a role of the microbiome in this case.

More work is needed to understand which members of the microbiome in bees play key roles in the metabolic conversion of secondary metabolites, but experiments by Kešnerová et al. (2017) on honeybees (*Apis mellifera*) colonised by single strains from the major bacterial phylotypes of the resident microbiome of corbiculate bees suggest a dominant role of Lactobacilli (clades "Firm-4" and "Firm-5" *sensu* Martinson et al. (2011)) and Bifidobacteria for the deglycosylation of flavonoid glycosides. The genomes of strains from the Firm-5 *Lactobacillus* clade contain a multitude of carbohydrate processing genes in bumblebees and honeybees (with a higher representation in the latter), including glycosidase hydrolases that could cleave sugars from glycosides (Ellegard et al. 2019). Bifidobacteria from bumblebees and honeybees similarly contain several glycoside hydrolases (Zheng et al. 2019). Lactobacilli and Bifidobacteria are therefore likely candidates for the deglycosidation of the unedone-8-*O*-*β*-D-glucoside observed in our experiment in the hindgut of bumblebees, but additional experiments are needed to verify this. Both *Lactobacillus* and *Bifidiobacterium* genomes show considerable variation in glycosidase hydrolase contents between strains (Ellegard et al. 2019; Zheng et al. 2019). This suggests the type of bacterial strains a bee is colonised with will influence processing of dietary secondary metabolites, and based on our findings, these strain level variations in the microbiome could, as a consequence, affect parasite persistence or establishment. Perturbation of the microbiome by pollutants, as has been shown for the effect of glyphosate and heavy metals on the honeybee microbiome (Motta et al. 2018; Rothman et al. 2019), could furthermore indirectly affect parasite success in the bee gut through changes in the metabolism of secondary metabolites by the microbiome.

Conversely, pollinators able to glycosylate secondary metabolites in the gut may have reduced antiparasitic benefits from them (but may benefit from lowered toxicity of the compounds to themselves (Despres et al. 2007; Heckel 2018)). To our knowledge, our detection of the glycosylation of unedone in the bumblebee midgut (including in microbiome deprived individuals) is the first record of this type of chemical modification of plant secondary metabolites in bees. Further studies are needed to determine how frequently glycosylation of nectar or pollen secondary metabolites occurs in bees and other pollinating insects. Uridine-diphosphate-glycosyl transferases (UGTs) catalyse the glycosylation of xenobiotics in insects and play a role in their detoxification (Despres et al. 2007; Ahn et al. 2012). UGTs are present in the genomes of honeybees, bumblebees, and solitary bees (Berenbaum & Johnson 2015), but only in few (2-12) copies compared to their presence in the genomes of other herbivorous insects such as lepidopterans (over 40 copies; Ahn et al. 2012). Mao et al. (2013) found that ingestion of the pollen secondary metabolite *p*-coumaric acid up-regulated the expression of UGTs in honeybees, consistent with their role in secondary metabolite detoxification in this bee species. Health trade-offs for pollinators between the benefits of detoxifying harmful secondary metabolites in the gut by glycosylation and the reduction of antiparasitic activity of glycosylated compounds as suggested in this study are plausible and deserve further attention. Under increased parasite pressure in the host environment, reduced glycosylation rates may be beneficial to limit parasite infections, as benefits from reduced parasite loads may outweigh the costs of damage from toxic secondary metabolites. Whether pollinators can fine-tune glycosylation in this manner in response to parasite risks remains to be investigated.

Lastly, both tree species studied here have major ecological significance as food plants for bumblebees. Strawberry trees (*A. unedo*) are the major autumn food source around the Mediterranean for *B. terrestris* (Rasmont et al. 2005), but also serve as late season foraging plants outside of its native range in urban environments, for example for gynes in the UK (Stelzer et al. 2010). *C. bombi* infections have high fitness costs for *B. terrestris* gynes (Brown et al. 2003), and therefore our observed protection of gynes against *C. bombi* by unedone from *A. unedo* may improve their chances for winter survival and successful nest establishment in the following season. Linden trees (*Tilia* spp.) are major nectar sources in urban environments and temperate deciduous forests (Somme et al. 2016; Koch & Stevenson 2017; Stevenson et al. 2020). Our work emphasises the potential benefits of trees for pollinators in urban environments, as some

species like strawberry and linden trees may not only provide an abundance of food, but also health benefits for pollinators. Our findings add to the evidence that plants with antiparasitic ("medicinal") activity for pollinators can offer a nature-based solution to maintaining or improving the health of wild pollinators. Positive effects for pollinator health could be achieved both through the conservation or restoration of key medicinal plant species in natural or semi-natural ecosystems (Koch et al. 2019), or through promoting medicinal plants in managed landscapes, such as agricultural field margins or urban green spaces (Giacomini et al. 2018; Folly et al. 2021). However, as our understanding of the impacts of nectar and pollen chemistry on wild pollinator health under field conditions remains limited, caution and more research are needed before guidance on landscape level manipulation of plant species composition for pollinator health can be given (Sutherland et al. 2020; Brown 2022).

Conclusion

We show that the antiparasitic activity of nectar secondary metabolites can be both increased and decreased during gut passage in a common bumblebee pollinator. This modulation of anti-parasitic activity can derive both from the host and the resident gut microbiome. Effects of secondary metabolites on pollinator parasites therefore cannot necessarily be extrapolated from *in vitro* studies, or studies of a single host-parasite system. Rather, an integrative view of the interaction of the hosts, parasites, secondary metabolites, and the resident gut microbiomes needs to be taken for a fuller understanding of the potential benefits of floral reward phytochemicals on pollinator health.

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Figure captions

Figure 1: Activity of dietary unedone and tiliaside against *C. bombi* in *B. terrestris*. **A:** Boxplot of faecal concentration of *C. bombi* in *B. terrestris* gynes 7 days post infection when feeding on control or unedone (3.79 mmol/l) diets. Image: *B. terrestris* gyne foraging on *A. unedo* at Kew Gardens, UK. **B:** Boxplot of faecal concentration of *C. bombi* in *B. terrestris* workers with pre-established infections after feeding for 7

days on control or tiliaside (9.88 mol/l) diets. Image: B. terrestris worker foraging on *Tilia tomentosa* flower at Kew Gardens, UK.

Figure 2: *In vitro* activity test of *Tilia* and *Arbutus* compounds against *C. bombi*, and chemical structures of compounds investigated in this study. **A:** Top: Structures of unedone and unedone-8-*O*-*β*-D-glucoside (numbered carbons for NMR resonances in Table S1). Bottom: *In vitro* assay comparing inhibition of *C. bombi* by unedone and unedone-8-*O*-*β*-D-glucoside at equimolar concentrations. Y-axis: *C. bombi* cell concentrations in culture after 7 days. Dose response curve (3 parameters log-logistic model) fitted to unedone responses, with 95% confidence intervals shaded around regression curve. Linear regression line fitted to unedone-8-*O*-*β*-D-glucoside responses shows no decline with increased dose. **B:** Top: Structures of tiliaside and the aglycone of tiliaside. Bottom: *In vitro* assay comparing inhibition of *C. bombi* by the aglycone of tiliaside and tiliaside at equimolar concentrations. Y-axis: *C. bombi* cell concentrations in culture after 7 days. Dose response curve (3 parameters log-logistic model) fitted to responses to both compounds, with 95% confidence intervals shaded around regression curve.

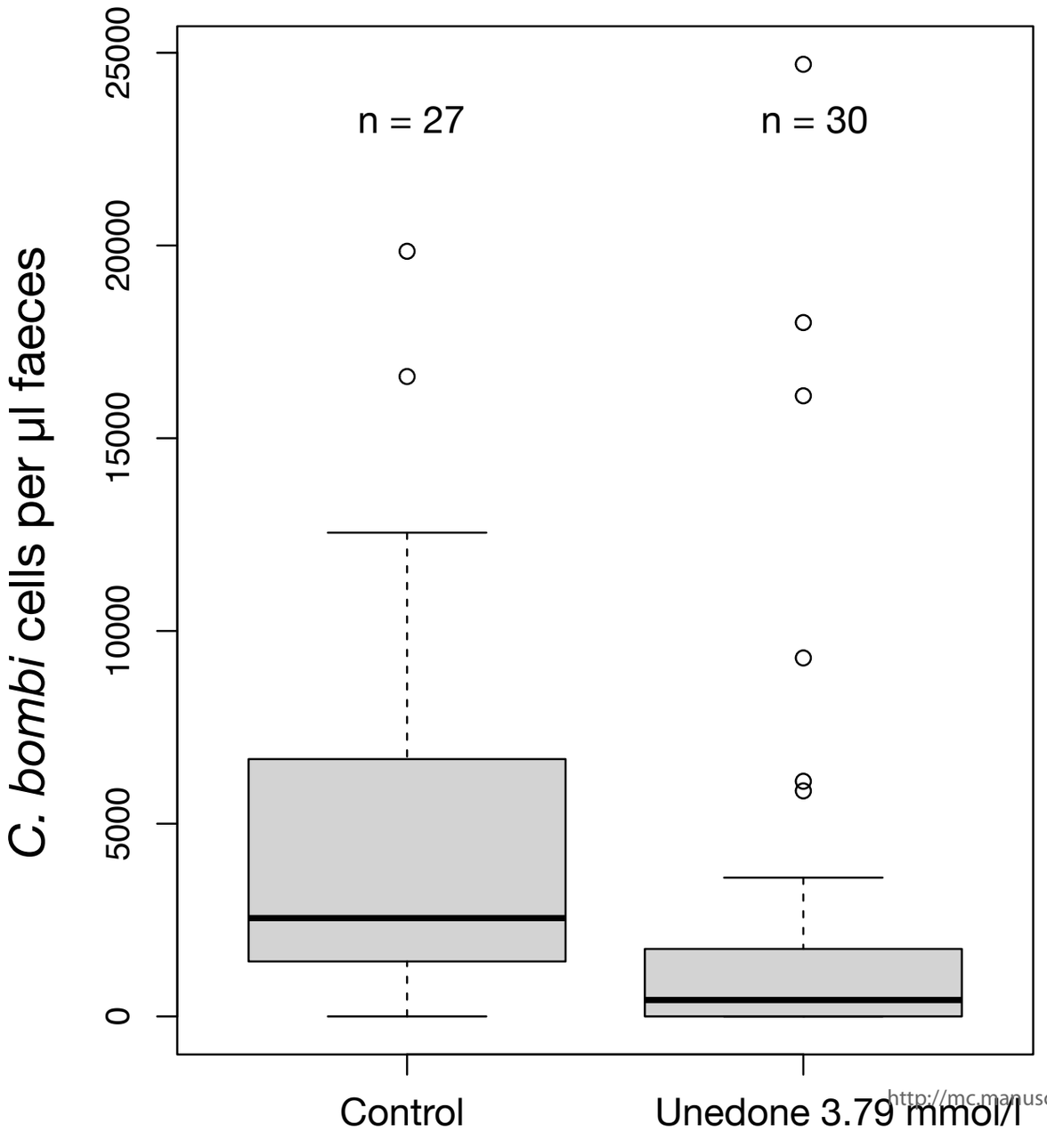
Figure 3: Conversion of unedone and tiliaside during gut passage in microbiome depleted or inoculated individuals. **A:** Boxplot of proportion of unedone to unedone-8-*O*-*β*-D-glucoside during gut passage in workers (top) and males (bottom) for microbiome depleted (-M: white boxes) or inoculated individuals (+M: grey shaded boxes). **B:** Schematic representation of proposed transformation of unedone during gut passage: unedone is ingested into the crop, transformed by the bumblebee into unedone-8-*O*-*β*-D-glucoside in the midgut and deglycosylated again into unedone by gut microbiome (grey rods) in the hindgut, where it can inhibit *C. bombi* parasites (red). **C:** Boxplot of proportion of the aglycone of tiliaside to tiliaside during gut passage in workers (top) and males (bottom) or microbiome depleted (-M: white boxes) or inoculated individuals (+M: grey shaded boxes).

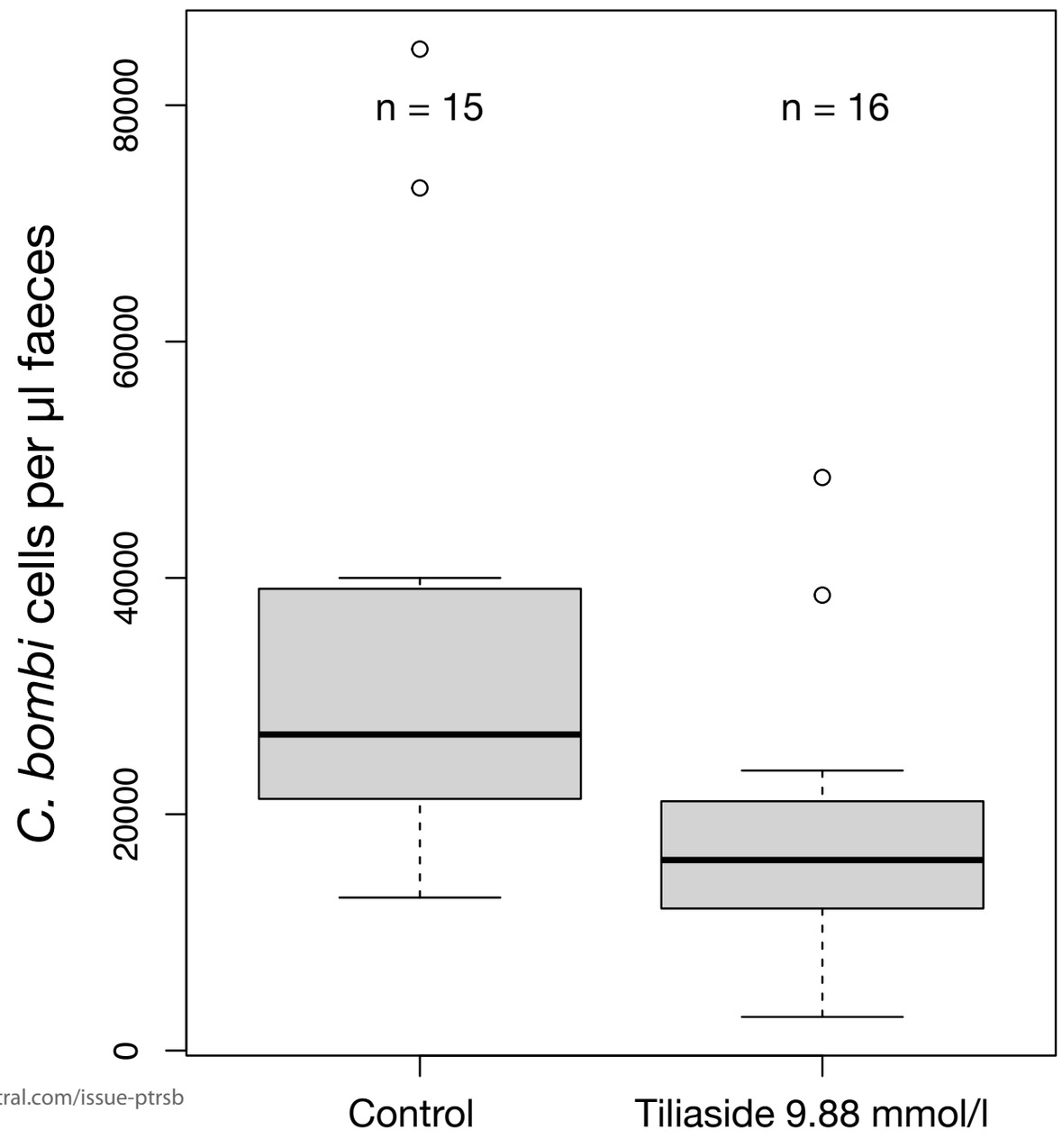
Supplementary File 1. Fig. S1: Conversion of tiliaside to the aglycone of tiliaside during gut passage; **Figs. S2-S7:** Structure and NMR spectroscopy results for unedone-8-*O*-*β*-D-glucoside. **Fig. S8:** NMR spectroscopy results for unedone. **Figs. S9-S10:** MS2 spectra for unedone-8-*O*-*β*-D-glucoside and unedone. **Figs. S11-S12:** Structural and NMR spectroscopy results for tiliaside and the aglycone of tiliaside. **Fig. S13:** MS2 spectra for tiliaside and the aglycone of tiliaside. **Tab. S1:** ^{1}H and ^{13}C NMR spectral data unedone-8-*O*-β-glucoside. **Tab. S2:** ^{1}H and ^{13}C NMR spectral data for tiliaside and the aglycone of tiliaside.

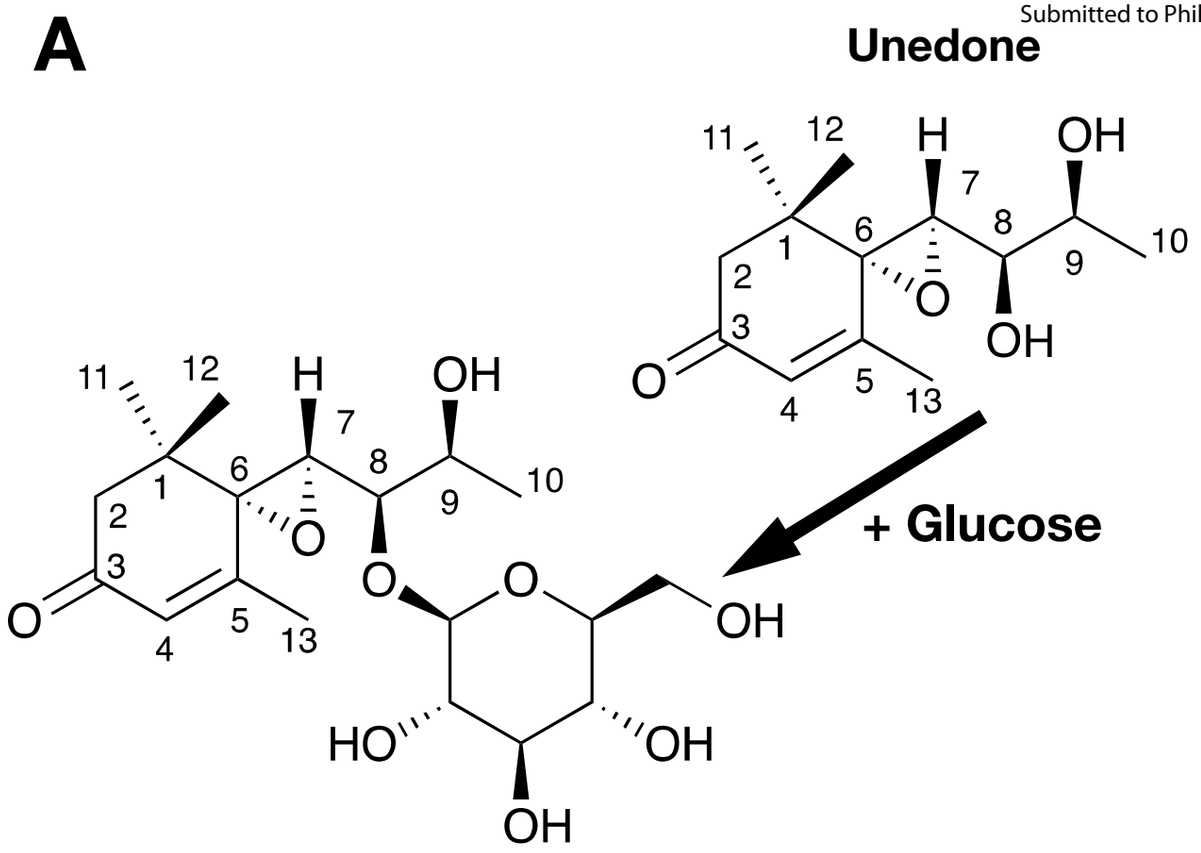
Supplementary File 2. Supplementary data

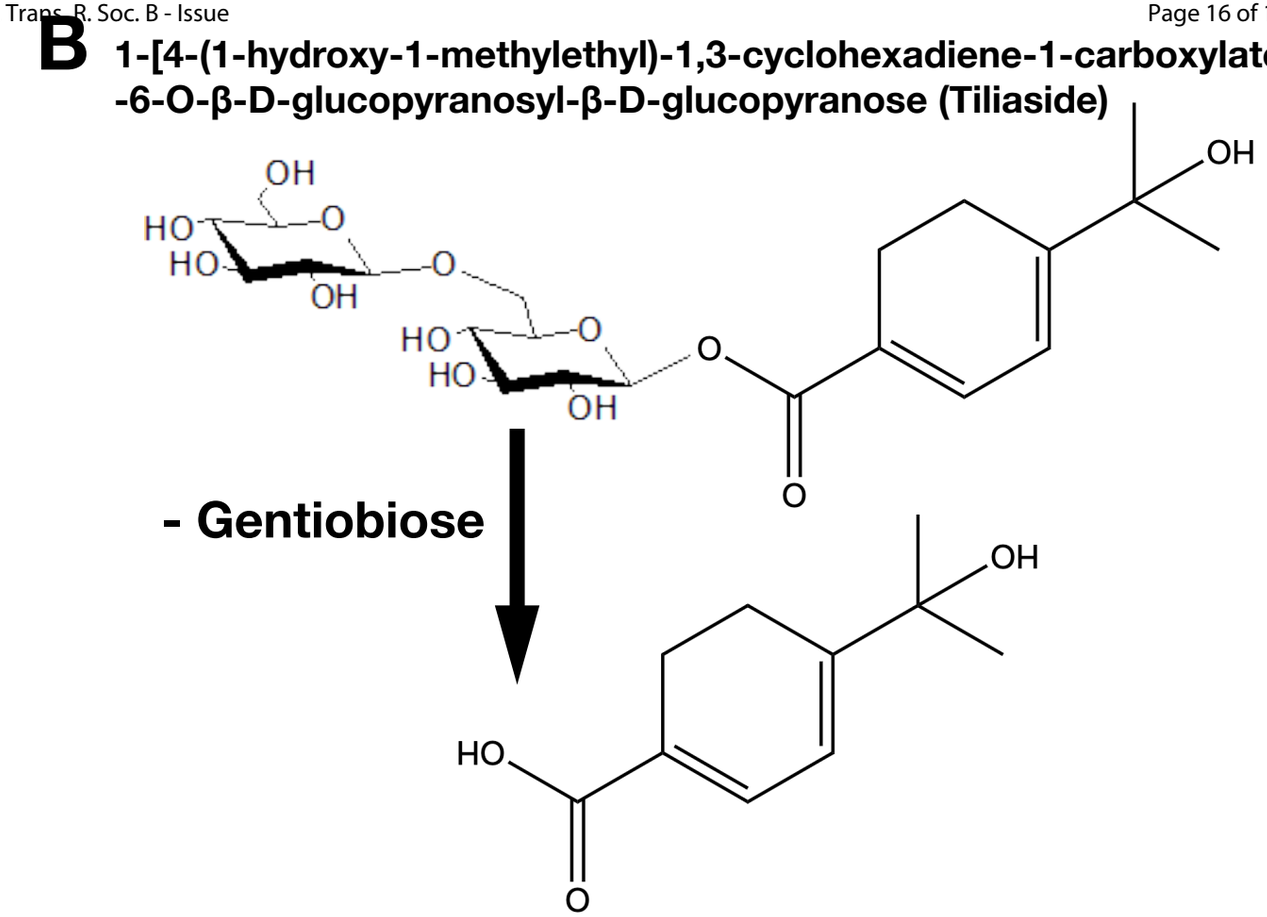
A

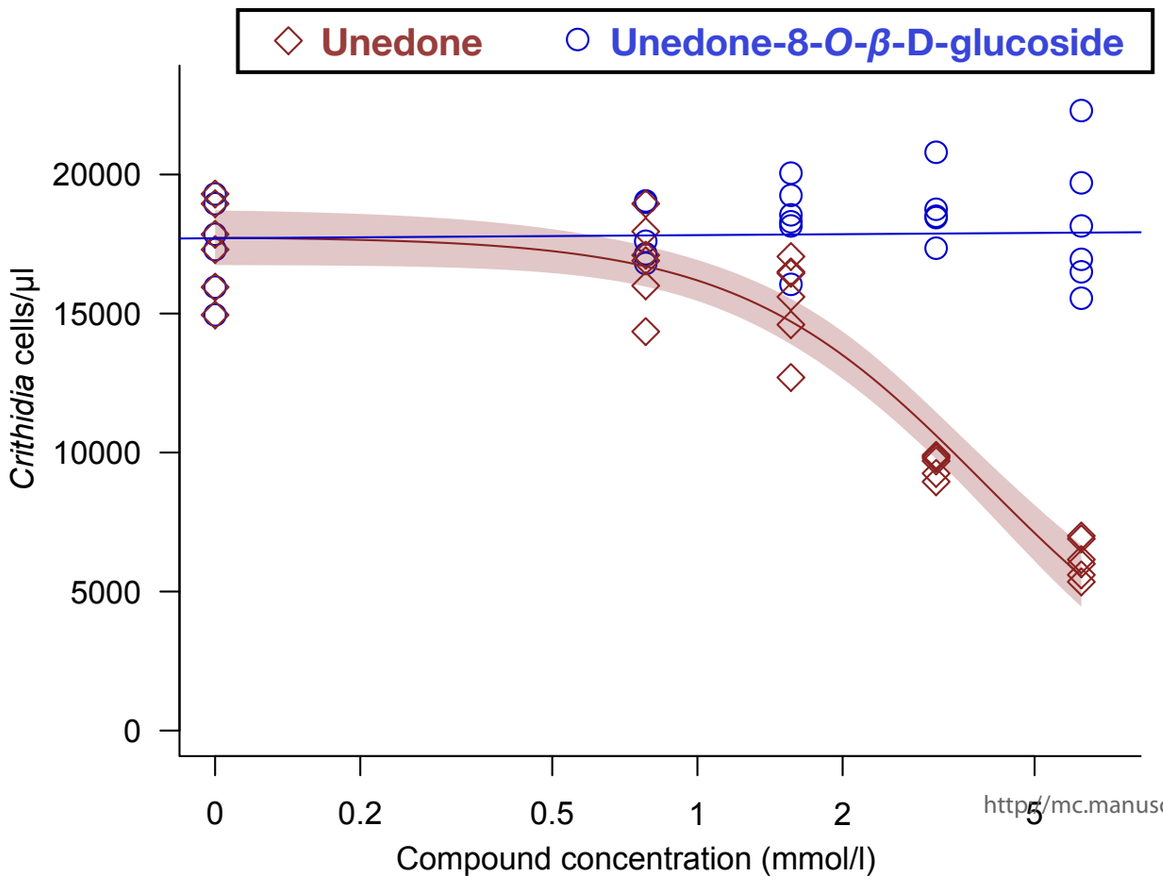
B

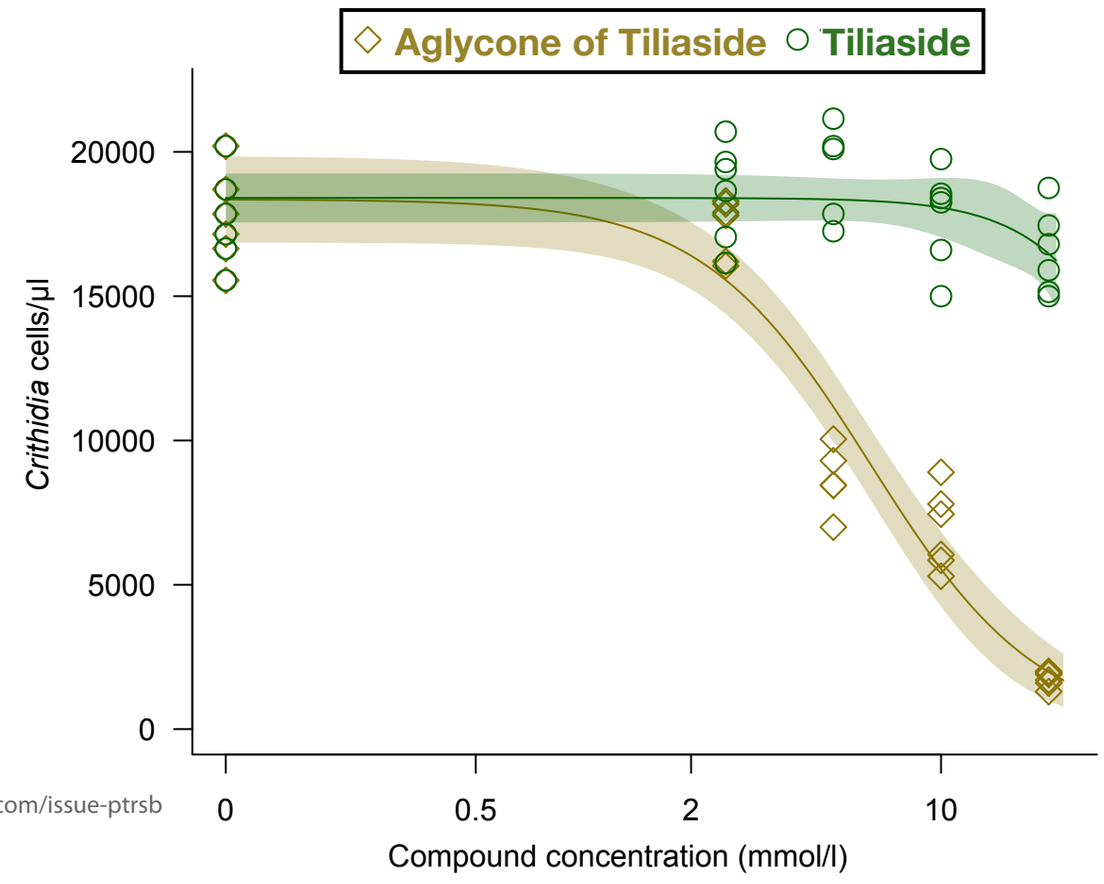


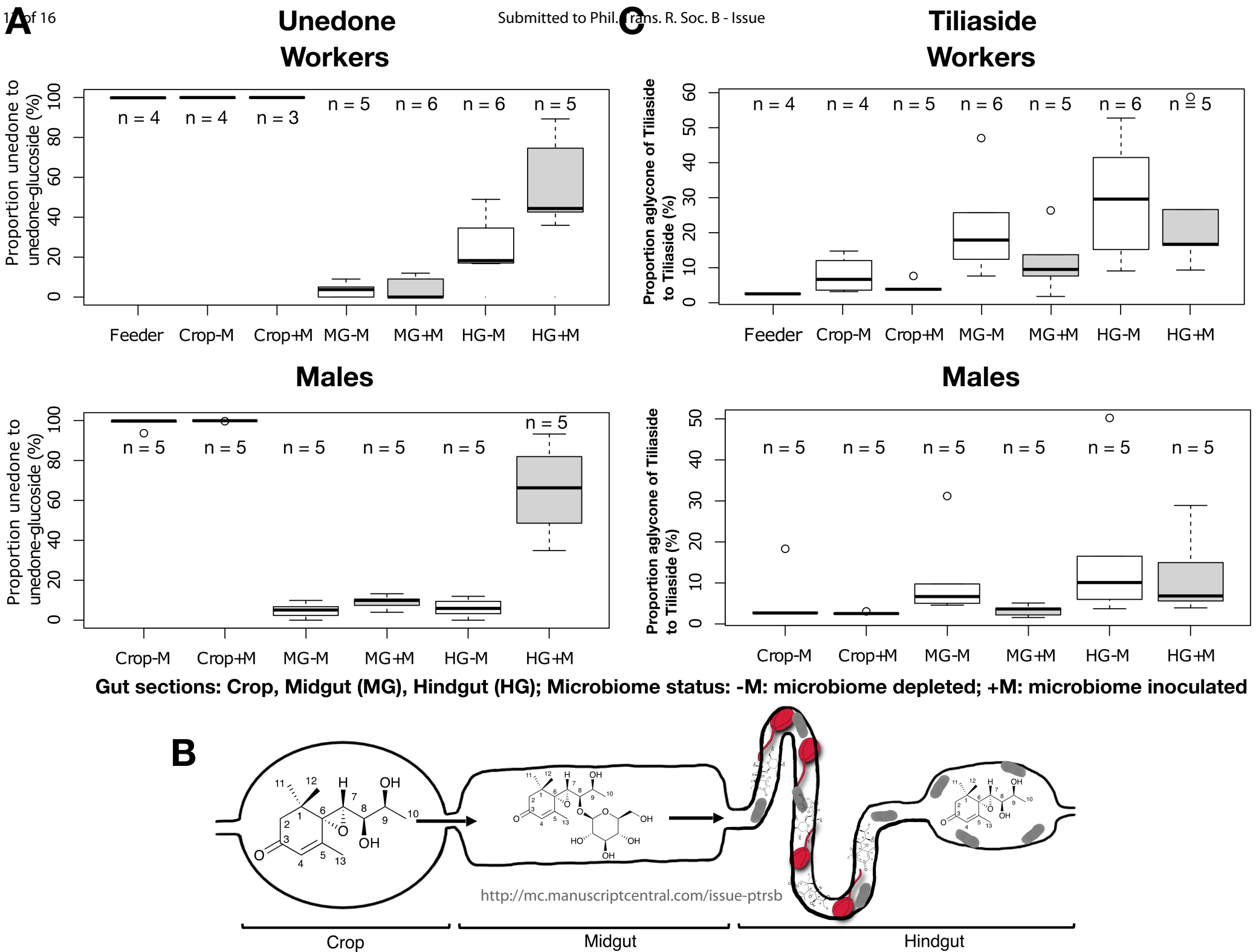











A
Unedone
Workers
Proportion unedone to unedone-glucoside (%)
0
20
40
60
80
100
n = 4
n = 4
n = 3
n = 5
n = 6
n = 6
n = 5
Feeder
Crop-M
Crop+M
MG-M
MG+M
HG-M
HG+M
C
Tiliaside
Workers
Proportion aglycone of Tiliaside to Tiliaside (%)
0
10
20
30
40
50
60
n = 4
n = 4
n = 5
n = 6
n = 5
n = 6
n = 5
Feeder
Crop-M
Crop+M
MG-M
MG+M
HG-M
HG+M
Males
Proportion unedone to unedone-glucoside (%)
0
20
40
60
80
100
n = 5
n = 5
n = 5
n = 5
n = 5
n = 5
Crop-M
Crop+M
MG-M
MG+M
HG-M
HG+M
Males
Proportion aglycone of Tiliaside to Tiliaside (%)
0
10
20
30
40
50
n = 5
n = 5
n = 5
n = 5
n = 5
n = 5
Crop-M
Crop+M
MG-M
MG+M
HG-M
HG+M
Gut sections: Crop, Midgut (MG), Hindgut (HG); Microbiome status: -M: microbiome depleted; +M: microbiome inoculated
B
http://mc.manuscriptcentral.com/issue-ptrsb
Crop
Midgut
Hindgut