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Report

Flagellum Removal by a Nectar Metabolite Inhibits Infectivity of a Bumblebee Parasite

Highlights

- Callunene from heather nectar inhibits the bumblebee parasite *Crithidia bombi*

- *C. bombi* anchors to the ileum epithelium using its flagellum

- Callunene removes the flagellum, resulting in reduced infectivity of *C. bombi*

- Heathland declines could reduce access to this natural antimicrobial for bumblebees

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In Brief

Koch et al. elucidate the first mechanism that explains how nectar secondary metabolites reduce infection by the common bumblebee parasite *Crithidia bombi*; exposure to callunene, a megastigmene from heather nectar, results in the loss of the parasite flagellum, leading to reduced infectivity.

CellPress

Flagellum Removal by a Nectar Metabolite Inhibits Infectivity of a Bumblebee Parasite

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SUMMARY

Plant phytochemicals can act as natural "medicines" for animals against parasites [1–3]. Some nectar metabolites, for example, reduce parasite infections in bees [4–7]. Declining plant diversity through anthropogenic landscape change [8–11] could reduce the availability of medicinal nectar plants for pollinators, exacerbating their decline [12]. Existing studies are, however, limited by (1) a lack of mechanistic insights into how phytochemicals affect pollinator diseases and (2) the restriction to few, commercially available chemicals, thereby potentially neglecting plants with the biggest antiparasitic effects. To rapidly identify plants with the greatest potential as natural bee medicines, we developed a bioactivity-directed fractionation assay for nectar metabolites. We evaluated 17 important nectar plants against the bumblebee pathogen *Crithidia bombi* (Trypanosomatidae) [13–17]. The most bioactive species was heather (*Calluna vulgaris*), the second most productive UK nectar plant [10]. We identified 4-(3-oxobut-1-enylidene)-3,5,5-trimethylcyclohex-2-en-1-one (callunene) from heather nectar as a potent inhibitor of *C. bombi*. Wild bumblebees (*Bombus terrestris*) foraging on heather ingest callunene at concentrations causing complete *C. bombi* inhibition. Feeding on callunene was prophylactic against infections. We show that *C. bombi* establishes infections by flagellar anchoring to the ileum epithelium. Short-term callunene exposure induced flagellum loss in *C. bombi* choanomastigotes, resulting in a loss of infectivity. We conclude that plant secondary metabolites can disrupt parasite flagellum attachment, revealing a mechanism behind their prophylactic effects. The decline of heathlands [18–21] reduces the availability of natural bee "medicine" and could exacerbate the contribution of diseases to pollinator declines.

RESULTS AND DISCUSSION

Discovery of a Potent Antiparasitic Secondary Metabolite (Callunene) from Heather Nectar

Plants produce a diversity of secondary metabolites in nectar, including alkaloids, flavonoids, and terpenoids, with a variety of ecological functions [22, 23]. Some of these metabolites exhibit antimicrobial activity and may have medicinal benefits for bees against their diseases [6, 12, 24, 25]. Understanding these interactions is urgent, as bees provide critical pollination services [26–29] but are threatened by interacting stressors including diseases [13, 29–34]. However, so far only a few, commercially available compounds have been tested against bee parasites, because the isolation of nectar metabolites in sufficient quantities for bioassays is challenging ([4, 6, 7]; but see [35]). In addition, their mechanism of action remains unknown. We developed a bioactivity-directed fractionation protocol to rapidly screen nectar metabolites from a variety of plant species and identify compounds with activity against the bumblebee gut parasite *Crithidia bombi*. *C. bombi* is a widespread and locally common parasite [15] that reduces bumblebee fitness by strongly decreasing colony-founding success in queens [17], and through decreased starvation tolerance in workers [16]. The parasite transmits between bumblebees through ingestion of cells shed in the feces of infected hosts and establishes infections in the gut of the host [16].

Using ethyl acetate extracts of monofloral honeys, we were able to partition nectar secondary metabolites from 17 key bee forage plants for bioassays against the parasite (see Key Resources Table). Our selection included 7 out of the top 12 dominant UK nectar plants in terms of the total weight of nectar sugar produced across the UK landscape, and as estimated by Baude et al. [10]: clover (*Trifolium*), ling heather (*Calluna vulgaris*), bell heather (*Erica*), dandelion (*Taraxacum* agg.), oilseed rape (*Brassica napus*), ivy (*Hedera helix*), and blackberry (*Rubus fruticosus* agg.).

Honey extracts varied in their inhibitory effects on *C. bombi* growth (Figure 1A). Complete inhibition (i.e., no growth *in vitro* after 5 days) was observed for extracts from *Calluna* heather (*Calluna vulgaris*) and viper's bugloss (*Echium vulgare*) monofloral honeys. Strawberry tree (*Arbutus unedo*) and linden (*Tilia* sp.) honey extracts also showed significant reduction in *C. bombi* growth, whereas for the remaining monofloral honey extracts,
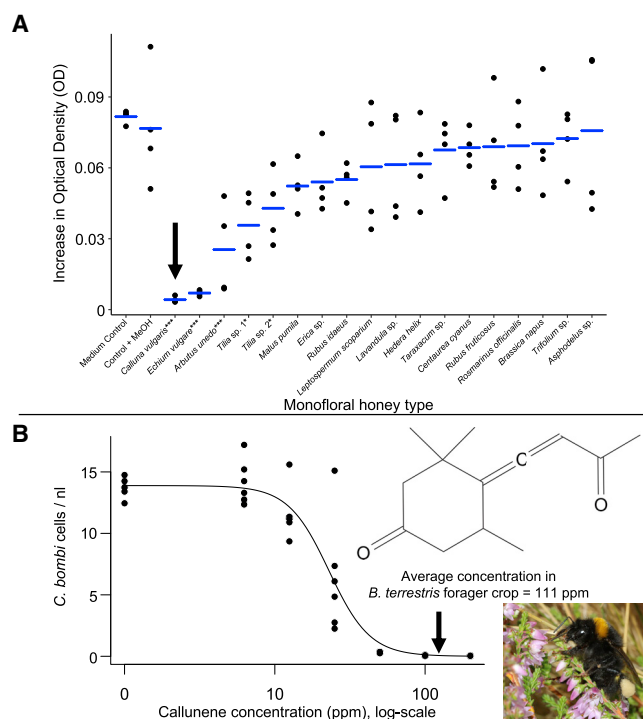
Figure 1. Discovery of a Potent Antiparasitic Secondary Metabolite (Callunene) from Heather Nectar

(A) Increase in optical density (OD) of *C. bombi in vitro* culture after 5 days when treated with ethyl acetate extracts of different monofloral honeys. Four replicates per treatment (black dots) and treatment mean (blue bars). Arrow marks *Calluna* heather extract. Medium control: growth in pure *C. bombi* medium; medium+MeOH: growth in *C. bombi* medium with 1% methanol. Asterisks following treatments indicate significantly lower increase in OD compared to medium+MeOH control, i.e., significant growth inhibition by the extract (Dunnett's test, ***$p < 0.001$, *$p < 0.05$).

(B) Dose-response curve for *C. bombi* cell concentration *in vitro* after 7 days of growth under different callunene concentrations (6 replicates per treatment). Right: structure of callunene (top) and *B. terrestris* foraging on *Calluna vulgaris* (bottom).

See also Figures S1, S2, and S3 and Table S1.

Table 1. Concentrations of Callunene in *C. vulgaris* Plant Samples and *B. terrestris* Wild and Laboratory Worker Gut Segments

Sample	Average (ppm)	Range (ppm)	N
C. vulgaris nectar	51.7	35.8–67.5	2
C. vulgaris honey	46.2	31.8–58.2	4
C. vulgaris pollen	0.6	0–1.9	3
C. vulgaris flower	ND	ND	2
C. vulgaris leaf	ND	ND	2
Crop wild	111	15.8–376.3	10
Mid- and hindgut wild	2.34	0.1–10.9	10
Crop (laboratory)	40.4	29.2–54.6	5
Midgut (laboratory)	0.1	0–0.4	6
Hindgut (laboratory)	0.35	0–0.9	6

Laboratory workers had fed on 1-fold heather honey extract for 7 days at the time of dissection. See also Figure S3.

We detected callunene in the nectar of wild *C. vulgaris* plants at concentrations comparable to those found in honey extracts (Table 1; Figure S3). This demonstrated *C. vulgaris* nectar as the natural source of callunene in monofloral heather honey. *B. terrestris* workers foraging on *C. vulgaris* also contained the compound in their honey crop, at an average concentration of 111 ppm (0.54 μmol/mL) (Table 1; Figure S3). Callunene is therefore consumed naturally by bumblebees foraging on *C. vulgaris* at concentrations higher than that required for complete *C. bombi* inhibition *in vitro* (Figure 1B). In contrast to the observed high concentration of callunene in the crop, the compound was, however, largely absent from the mid- and hindgut of foragers (Table 1). Callunene was not detected in leaves and flowers, and only in one pollen sample at a very low concentration, possibly because of nectar contamination during sampling (Table 1). This suggests that the metabolite is specific to *C. vulgaris* nectar.

Callunene has previously been described from heather honey [36, 37], but here we document both *C. vulgaris* nectar as its natural source for the first time and demonstrate its potent bioactivity against *C. bombi*. The presence of callunene in honey of the distantly related leatherwood (*Eucryphia lucida*: Cunoniaceae) from Tasmania [38] suggests that this compound is more widespread in nectar than currently recognized and could impact pollinator diseases in broader ecological and geographic contexts than those studied here. We note that *Calluna* heather honey has also been found to exhibit strong activity against human and equine pathogens, even surpassing manuka honey [39, 40], but so far without identification of its main active principle. Our characterization of callunene as the potent antimicrobial could thus have medicinal implications beyond our pollinator-focused research, and its activity against human or livestock pathogens should be investigated. The allenic moiety in callunene is generally uncommon in natural products, but where it does occur it is often associated with high biological activity, including strong antibiotic effects [41]. This is possibly due to the high reactivity of its two adjacent (cumulated) carbon-carbon double bonds [41].

Our results show that monofloral honeys provide a good basis for screening the large diversity of nectar metabolites for their
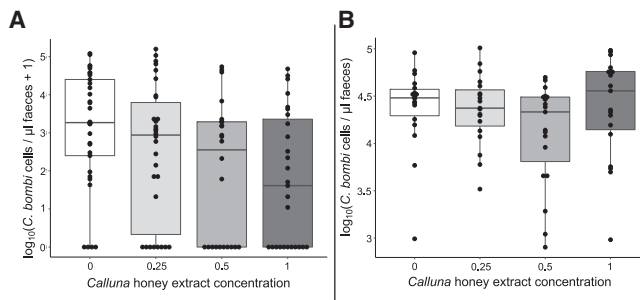
parasite growth was within the range of the controls (Figure 1A). Here we focused further experiments on *C. vulgaris*, the plant with the most potent inhibitory honey extract and the second largest contributor to nectar provision in the United Kingdom [10]. We combined chromatographic fractions with significant inhibitory activity from the first stage of our bioactivity-directed fractionation of the *Calluna* honey extract (Figure S1A) and isolated 9 target molecules for re-isolation, using semi-preparative high-performance liquid chromatography (HPLC), of which only one showed strong, significant *in vitro* activity against *C. bombi* (Figure S1B). Nuclear magnetic resonance (NMR) spectroscopy of the compound purified from this fraction, together with published data [36], was used to elucidate the structure as 4-(3-oxobut-1-enylidene)-3,5,5-trimethylcyclohex-2-en-1-one (Figures 1B and S2; Table S1). We assign the trivial name callunene to this compound. The IC_{50} of callunene against *C. bombi* was estimated at 23 ppm (113 μM) (Figure 1B), when testing callunene concentrations from 0 to 200 ppm (0–980 μM).

Figure 2. Heather Honey Extract Prevents *Crithidia bombi* Infections in Bumblebees but Does Not Cure Existing Ones

(A) Boxplot of *C. bombi* infection load in *B. terrestris* workers that fed on different heather honey extract concentrations (1 = 1-fold honey concentration) 2 days prior to infection and during the 7 days after infection. *C. bombi* cell concentrations were assessed in fecal samples 7 days after infection.

(B) Boxplot of *C. bombi* infection load in *B. terrestris* workers that carried a 7-day-old infection at the start of the heather honey extract treatment. Workers with established infections were fed on different heather honey extract concentrations (1 = 1-fold honey concentration). *C. bombi* cell concentrations were assessed in fecal samples after 7 days on the heather honey extract diet.

activity against bee parasites. As demonstrated here for heather, monofloral honeys often contain similar secondary metabolites to the original nectar (see also, e.g., [42–44]) and so are a good proxy for discovery and isolation of natural antiparasitic compounds for bees. We demonstrated that bioactivity-directed fractionation is an efficient method to identify active principles in floral rewards against pathogens. In the future, this approach can be used to screen a wider taxonomic and geographic range of monofloral honeys to identify other antimicrobial nectar compounds against parasites of pollinators. It could also be extended to discover antiparasitic compounds in pollen, for example, the unknown active principle behind strong *C. bombi* inhibition in the North American bumblebee *Bombus impatiens* by sunflower pollen [45].

Heather Honey Extract Prevents *Crithidia bombi* Infections in Bumblebees but Does Not Cure Existing Ones

B. terrestris workers that fed on *Calluna* honey ethyl acetate extracts 2 days before and immediately after parasite inoculation had lower infection probabilities with increasing extract concentrations (generalized linear model [GLM], χ^2 = 4.3, p = 0.037) (Figure 2A), with a significant colony effect on infection outcome (GLM, χ^2 = 14.7, p = 0.04). Higher extract concentrations also resulted in lower overall infection intensities (F(1,105) = 8.2, p = 0.0052) (Figure 2A), again with colony identity being a significant additional factor determining parasite load (F(7, 105) = 9.0, p = 1.2×10^{-8}). In contrast, we found no effect on *C. bombi* parasite loads when *B. terrestris* workers with established infections fed on *Calluna* honey extracts at up to 1-fold honey concentration for 7 days (F(1,70) = 0.002, p = 0.96), and no existing infections were cleared (Figure 2B); colony identity did not affect the infection outcome (F(3,70) = 0.68, p = 0.56). This suggested a prophylactic but not curative effect on established infections of the extract containing callunene. Analysis of dissected gut fragments from *B. terrestris* workers that had fed on the 1-fold *Calluna* honey extract for 7 days revealed that callunene occurred in the honey

crop at concentrations comparable to the original honey or *C. vulgaris* nectar (Table 1; Figure S3). In contrast, the midgut and hindgut had either very low or undetectable levels, mirroring our findings in field-collected animals (Table 1; Figure S3) and suggesting the compounds had been metabolized. Microscopic examination of dissected and homogenized gut segments of *B. terrestris* workers fed on the sugar water control diet showed that *C. bombi* infections were restricted to the hindgut (ileum and rectum) of the host, with the highest concentration in the ileum (Figure S4). *C. bombi* would therefore be exposed to high concentrations of callunene only during passage through the crop, but not when an infection is already established in the hindgut.

The fate of plant secondary metabolites in animal hosts after ingestion is often poorly known, but crucial to understanding their effects [46]. As documented by Gorbunov [47] for *Bombus pascuorum*, we show that active infections with *C. bombi* in *B. terrestris* are restricted to the hindgut. This location could shield *C. bombi* from exposure to antimicrobial phytochemicals, if these are either absorbed or degraded by the host or associated gut microorganisms prior to reaching the hindgut. Indeed, we observed that even though callunene is taken up by bumblebee foragers in the field at concentrations well above levels required for total inhibition of *C. bombi* growth *in vitro*, it does not reach the hindgut at inhibitory concentrations. We note that *C. bombi* may also benefit in other ways from establishing infections in the hindgut, as it could, for example, facilitate dispersal via excretion of rectal contents, or provide access to essential nutrients such as amino acids in the hindgut lumen.

Processing of phytochemicals during the gut passage could in part explain the current discrepancies between studies examining *C. bombi*—phytochemical interactions *in vitro* and *in vivo*, or different results for antimicrobial activity in separate *in vivo* studies if hosts differ in their ability to degrade secondary metabolites [6, 25, 48]. Similar to our findings for callunene, Palmer-Young et al. [49], for example, found strong inhibition of *C. bombi* by 50 ppm eugenol *in vitro* but no effect on *C. bombi* infections in *Bombus impatiens* feeding on a diet containing eugenol at the same concentration, possibly due to the degradation or absorption of eugenol in the midgut. We stress that future studies will need to examine the way phytochemicals are processed in the bee gut in more detail, either by the host or the gut microbiome [50, 51].

We hypothesized that harmful effects of short-term exposure to callunene on *C. bombi* during the passage through the anterior gut (crop) may explain the reduced likelihood of infections in *B. terrestris* feeding on callunene-containing extracts prior and immediately after parasite exposure. Previously, Michaud et al. [52] showed that short-term exposure to the nectar metabolite aucubin before ingestion can subsequently reduce *C. bombi* infection loads in *Bombus impatiens*. Similarly, Rothchild et al. [53] found changes in cell morphology and decreased viability of *C. bombi* cells after short time exposure to thymol *in vitro*, but only at a concentration several times higher than naturally occurring in nectar.

Callunene Removes the Flagellum; *Crithidia* Loses Infectivity

To determine the mechanism behind the prophylactic effect of callunene, we microscopically examined its direct effect on
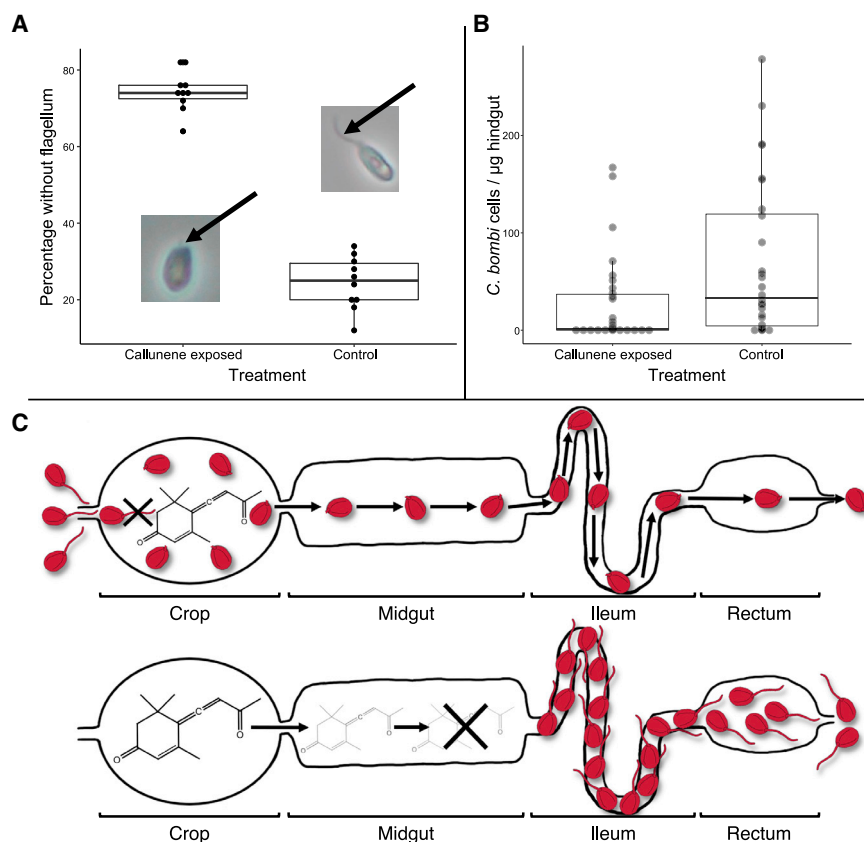
Figure 3. Callunene Removes the Flagellum; *Crithidia* **Loses Infectivity**

(A) Boxplot of percentage of *C. bombi* cells with flagellum reduced to a small stump or entirely absent (see left inserted picture and Video S1) after 90 min in medium with 110 ppm callunene versus in control medium without callunene. Inserted picture on the right shows *C. bombi* choanomastigote cell with typical, long flagellum for comparison. 10 replicates per treatment.

(B) Boxplot of *C. bombi* concentrations per μg of hindgut weight 7 days after infection with cells either treated for 60 min with 200 ppm callunene or with control medium without callunene.

(C) Schematic representation of the proposed interaction between *C. bombi* and callunene along the bumblebee intestinal tract. Upper: prophylactic effect of callunene: *C. bombi* cells are ingested and exposed to callunene from ingested nectar in the crop. After the loss of a functional flagellum, cells lose motility and the ability to attach to the ileum epithelium, and are passed through the gut without establishing an infection. Lower: callunene fails to cure infections: ingested callunene is degraded or absorbed during passage through the midgut. Infecting *C. bombi* cells are sheltered from callunene, attaching to the ileum epithelium with their flagellum, or swimming freely in the rectum prior to expulsion and dispersal.

See also Figure S4 and Videos S1 and S2.

C. bombi cells. Short time exposure (90 min) *in vitro* to callunene at 110 ppm (the mean concentration of this compound in the crop) resulted in the loss of the flagellum of *C. bombi* choanomastigote cells when compared to untreated controls (Wilcoxon rank-sum test, W = 100, p = 0.00018) (Figure 3A; Video S1). We note that the doubling time of *C. bombi* has been estimated at 10–16 h [54], and therefore our exposure time frame excludes inhibition of flagellum formation in new cells as an explanation for the observed absence of the flagellum in treated cells. Video S1 furthermore shows the gradual loss of a functional flagellum of an individual *C. bombi* cell within under 20 min after treatment with 200 ppm callunene on a wet-mount microscopic slide. We therefore examined the possible role of the flagellum in the infection process *in situ*. *C. bombi* cells were found to adhere to the ileum wall of *B. terrestris* workers with their flagellum and swam freely in the rectum (Video S2). This is the first demonstration that the flagellum of *C. bombi* is essential to the establishment of infections in the ileum of the host. *C. bombi* parasite cells that are newly ingested by bumblebee foragers in the field are likely exposed to concentrations of callunene in the crop for at least the duration of the foraging bout, during which nectar is accumulated in this part of the gut. To test the effect of this type of exposure with the associated flagellum loss on infection establishment, we first exposed *C. bombi* cells *in vitro* to 200 ppm callunene for 60 min (representing an average *B. terrestris* foraging bout duration [55], and with a callunene concentration within the range of the values recorded by us for wild *B. terrestris* crops) immediately before ingestion by bumblebee

workers. This exposure led to a significantly reduced infection rate in *B. terrestris* workers relative to controls (GLM, χ^2 = 5.6, p = 0.018), and an overall lower infection load ($F_{(1,54)}$ = 7.4, p = 0.0089) after 7 days (Figure 3B), with no significant impact of colony identity on infection rate (GLM, χ^2 = 0.4, p = 0.81) nor infection intensity ($F_{(2,54)}$ = 0.17, p = 0.85). This demonstrated that short time exposure to callunene at concentrations and durations likely encountered by the parasite in the crop of foraging bumblebees can prevent it from establishing an infection.

The flagellum plays an important role for other trypanosomatids in the infection of insects [56]. *Crithidia fasciculata* attaches to the hindgut wall of mosquitoes with its flagellum [57], and the human pathogenic *Leishmania mexicana* inserts its flagellum between the midgut microvilli for attachment in its sandfly vector (*Lutzomyia longipalpis*) [58]. *Leishmania mexicana* knockout mutants without functional flagellum fail to infect sandflies [59]. We note, however, that not all trypanosomatids attach to the gut with their flagellum; for example, the honeybee parasite *Lotmaria passim* adheres to the rectum with non-flagellated "spheroid" cells [60], which could lead to variation across host-parasite systems in the protective properties of metabolites. In addition, we point out that *C. bombi* genotypes can vary in their resistance to phytochemicals [25], and our study was restricted to a single isolate. Future work should therefore investigate the effects of callunene on additional isolates of *C. bombi* to test whether some naturally occurring genotypes have evolved resistance to this nectar compound.

Our results suggest that plant metabolites such as callunene can disrupt essential flagellar functions such as motility and attachment, thereby preventing hosts from becoming infected. This offers, for the first time, a mechanistic basis for our understanding of prophylactic effects of nectar metabolites on the infection success of trypanosomatid parasites in animal hosts. Disruption of the flagellum by plant metabolites could be an important target to identify new antiparasitic compounds from natural sources.

Declining Heathlands: Are Bees Losing a Key Medicinal Plant?

Heather (*C. vulgaris*) is a major foraging resource for bumblebees and other pollinators [10, 61–65]. In a study estimating the amount of nectar sugar produced by flowering plants in the United Kingdom, *C. vulgaris* was ranked at 2nd place by total yearly amount [10]. Our results suggest that beyond the important nutritional role of *C. vulgaris* for bees, feeding on *C. vulgaris* nectar can also provide medicinal benefits by preventing parasite infections. Henson et al. [66] intriguingly detected few *C. bombi*-infected bumblebees in a survey of bumblebee parasites in heathland sites of southern England, but we stress that the effects of *C. vulgaris* nectar metabolites on the transmission of bee parasites need to be studied further under field conditions to ascertain whether their presence reduces infections at the landscape level. Alarmingly, heathlands are declining globally due to land-use changes and eutrophication [18–21]. The disappearance of heathlands may therefore lead to the loss of a key medicinal plant for bees, and our results emphasize the importance of their conservation.

Land-use change and climate change are resulting in global declines of plant diversity more broadly [11, 67, 68]. In light of the growing threats to wild animal populations from pathogens, including emerging diseases [69, 70], and synergistic detrimental effects between diseases and man-made stressors such as pesticides [71–74] or climate change [75], there is an urgent need to identify those natural plant medicines that can mitigate wildlife diseases and act to protect them.

Conclusions

We identified callunene from heather (*Calluna vulgaris*) nectar as a potent inhibitor of the common bumblebee gut parasite *Crithidia bombi* by developing a bioactivity-directed fractionation screen for nectar metabolites. Callunene had prophylactic but not curative effects for bumblebees (*B. terrestris*) against *C. bombi* (Figure 3C). We show that *C. bombi* uses the flagellum for attachment in the gut, and short-term exposure to callunene (such as that likely experienced by *C. bombi* under field conditions when passing through the crop) leads to flagellum removal and diminished infectivity. Our work provides the first mechanistic basis for our understanding of anti-parasitic effects from nectar metabolites for bees. As heather is a major foraging plant for European bees, it is likely of major importance for bee disease dynamics. Consequently, continued anthropogenic decline of heathlands may lead to the loss of a major medicinal plant for pollinators.

STAR★METHODS

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
Bombus terrestris subsp. *audax* (Harris, 1776): worker field samples	Foraging on *Calluna vulgaris* at Wimbledon Common, Greater London, UK; 51°26'29.1"N 0°13'48.1"W	NA
Calluna vulgaris (L.) Hull: nectar, pollen, flowers, leaves	Growing wild at Wimbledon Common, Greater London, UK; 51°26'29.1"N 0°13'48.1"W	NA
Calluna vulgaris (ling heather) honey for extracts of first *in vitro* screen	Bienenhonig Rüdiger Feldt, Eichede, Germany	NA
Calluna vulgaris (ling heather) honey for extracts of *in vivo* experiments, bioactivity-guided-fractionation, and callunene isolation	Afon Mel, New Quay, Wales, UK	NA
Centaurea cyanus (cornflower) honey	Bienenhonig Rüdiger Feldt, Eichede, Germany	NA
Leptospermum scoparium (manuka) honey	Watson & Son, New Zealand	NA
Trifolium sp. (Clover) honey	Bienenhonig Rüdiger Feldt, Eichede, Germany; honey origin: New Zealand	NA
Arbutus unedo (strawberry tree) honey	Wild about Honey, Portugal	NA
Lavandula sp. (lavender) honey	Bienenhonig Rüdiger Feldt, Eichede, Germany; honey origin: France	NA
Erica sp. (bell heather) honey	Bienenhonig Rüdiger Feldt, Eichede, Germany; honey origin: Spain	NA
Brassica napus (rape) honey	Bienenhonig Rüdiger Feldt, Eichede, Germany	NA
Echium vulgare (viper's bugloss) honey	J. Friend and Co., New Zealand	NA
Rubus fruticosus (bramble) honey	Bienenhonig Rüdiger Feldt, Eichede, Germany	NA
Taraxacum sp. (dandelion) honey	Bienenhonig Rüdiger Feldt, Eichede, Germany	NA
Hedera helix (ivy) honey	Beeactive, UK; honey origin: Ireland	NA
Asphodelus sp. (asphodel) honey	Rau, Italy	NA
Tilia sp. (lime/linden) honey 1	Ogilvy's, UK; honey origin: Serbia	NA
Tilia sp. (lime/linden) honey 2	Tesco, UK; honey origin: Romania	NA
Rosmarinus officinalis (rosemary) honey	Bienenhonig Rüdiger Feldt, Eichede, Germany; honey origin: Spain	NA
Malus pumila (apple) honey	Bienenhonig Rüdiger Feldt, Eichede, Germany	NA
Rubus idaeus (raspberry) honey	Bienenhonig Rüdiger Feldt, Eichede, Germany	NA
Chemicals, Peptides, and Recombinant Proteins		
Callunene: 4-(3-oxobut-1-enylidene)-3,5,5-trimethylcyclohex-2-en-1-one	Isolated from heather (*Calluna vulgaris*) honey (Afon Mel, New Quay, Wales, UK)	PubChem CID: 527058; InChI = 1S/C13H16O2/c1-9-7-11(15)8-13(3,4)12(9)6-5-10(2)14/h5,7H,8H2,1-4H3
Methanol	Fisher	A456-212, CAS:67-56-1
Ethyl acetate	Fisher	E/0850/17, CAS: 141-78-6
Experimental Models: Organisms/Strains		
Bombus terrestris subsp. *audax* (Harris, 1776): colonies, workers for experiments	Biobest, Belgium; through Agralan, UK	https://www.agralan-growers.co.uk/bumblebees-for-pollination/research-hives.html SKU: BB121040-CF1
Crithidia bombi Lipa and Triggiani (1988) [14]	Isolated from faeces of *Bombus terrestris audax* queen, March 2017; Royal Botanic Gardens, Kew, UK	strain 17.01
Software and Algorithms		
R 3.5.1; R – car package; R - drc package, R - multicomp package	The R Project for Statistical Computing	https://www.r-project.org/

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Hauke Koch (h.koch@kew.org). A standard of callunene isolated from *Calluna* honey can be provided upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Crithidia bombi

An *in vitro* culture of *Crithidia bombi* was produced from faeces of an infected *Bombus terrestris audax* queen collected at the Royal Botanic Gardens, Kew in March 2017. The culture was initially established through a serial dilution of faeces in standard *Crithidia* liquid culture medium containing a "Mäser-mix" of antibiotics to supress bacterial and fungal growth (see Salathé et al. [54], for medium and antibiotic composition) and incubated for 7 days at 28°C and 3% CO_2. We examined cultures for *Crithidia* growth and contaminants at 640-fold magnification under a phase contrast microscope (Zeiss Photomicroscope III; Carl Zeiss AG, Germany). An uncontaminated culture was subsequently passaged two times in antibiotic-free liquid *Crithidia* medium (each time examined microscopically for contaminants) and cryopreserved for future experiments at −80°C with 15% glycerol. All *in vitro* experiments were conducted using standard *Crithidia* liquid medium [54] in 96 well tissue culture plates (Eppendorf, Germany), incubated at 28°C and 3% CO_2.

Bombus terrestris

Bombus terrestris audax colonies were purchased from Biobest (Belgium) and maintained under 24°C in darkness. A total of 15 colonies were used in the experiments (8 colonies for the experiment in Figure 2A, 4 colonies for the experiment in Figure 2B, and 3 colonies for the experiment in Figure 3B). Faeces of 5 workers from each colony was screened microscopically (640-fold magnification, phase contrast microscope: Zeiss Photomicroscope III; Carl Zeiss AG, Germany) to ensure colonies were not infected with *C. bombi*. Colonies were fed polyfloral, honeybee collected pollen (Biobest, Belgium) and Biogluc (Biobest, Belgium) sugar syrup. For infection experiments, adult workers were taken from the colonies and maintained individually in 350 mL inverted PET smoothie cup cages (packpack, Germany) on Apiinvert (Südzucker, Germany) sugar syrup diluted to 50% (w/w) with distilled water (Milli-Q; Sigma, St. Louis, MO), or on otherwise experimentally specified diets. A round, 90 mm cellulose filter paper piece (Whatman, UK) was placed at the bottom of each cage. Polyfloral pollen (see above) was provided *ad libitum*.

Wild *B. terrestris audax* foragers were collected on *C. vulgaris* at Wimbledon Common (Greater London, United Kingdom) in July/August 2018 with permission from Conservation Officer Peter Haldane.

Calluna vulgaris

Nectar, pollen, leaf and flower samples of *C. vulgaris* were taken from naturally growing, mature plants on a lowland heathland site (Wimbledon Common, Greater London, United Kingdom) in July/August 2018 with permission from Conservation Officer Peter Haldane.

METHOD DETAILS

In vitro testing of monofloral honey extracts

We extracted 18 different monofloral honeys of 17 bee forage plant species (see Key Resources Table) with ethyl acetate (Fisher Scientific, Leicestershire, UK). 5 g of each honey was first dissolved in 10 g of ultrapure water (Milli-Q; Sigma, St. Louis, MO) and shaken with 10 g of ethyl acetate until an emulsion was formed. Emulsions were left to separate for 24 hours in darkness at room temperature. The ethyl acetate layer was subsequently removed and dried down in a centrifugal evaporator (Genevac EZ-2; SP Scientific, Stone Ridge, NY) under vacuum at 50°C. Ethyl acetate was chosen because it extracts a broad range of plant secondary metabolites [76], while almost completely removing sugars [77] that could influence *in vitro* tests with *C. bombi* [78]. Dried extracts were dissolved in 50 μl methanol and mixed with 4.45 mL of *Crithidia* growth medium for *in vitro* testing.

All *in vitro* experiments were conducted using standard *Crithidia* liquid medium [54] in 96 well tissue culture plates (Eppendorf, Germany), incubated at 28°C and 3% CO_2. In each well, 20 μl of a 1000 cells/μl *C. bombi* culture were mixed with 180 μl of test medium. Monofloral honey extracts were thus tested at the equivalent of 1-fold honey concentration. All outermost wells were filled with sterile water to prevent edge effects due to evaporation of the medium. Growth was evaluated using a microplate reader (Infinite M200, Tecan Life Sciences, Switzerland), monitoring optical density (OD) at 620 nm, after shaking plates for 60 s. For all *in vitro* screens, culture media were sterile filtered with syringe filters (polyethersulfone membrane, pore size 0.2 μm; Whatman, GE Healthcare, Chicago, IL) after addition of test extracts. For each screen, we included controls with *C. bombi* growing in pure culture medium and medium with 1% methanol. We tested for significant inhibition of all extracts with an ANOVA and a subsequent post hoc Dunnett's test, comparing each treatment to the medium with 1% methanol control, using the multicomp package [79] in R 3.5.1 [80].

Bioactivity-directed-fractionation

Calluna heather honey (Afon Mel, New Quay, Wales, UK) was extracted by liquid-liquid extraction with ultrapure water and ethyl acetate. The water layer was partitioned 3 times with ethyl acetate, in each step the ethyl acetate layer was removed, and the aqueous

layer extracted again with an equal volume of ethyl acetate. The combined ethyl acetate extracts were dried in a rotary evaporator until complete removal of the solvent.

The ethyl acetate extract of 50 g of honey was fractionated by flash chromatography (Biotage Isolera One; Biotage, Sweden) using a SNAP Ultra C18 cartridge. A linear gradient from 10% methanol in water to 100% methanol was run at a flow rate of 50 ml/min for 10 column volumes. The eluate was split into 10 subsequent fractions of equal volume and dried down (Genevac EZ-2; SP Scientific, Stone Ridge, NY) for *in vitro* testing at the equivalent of 3.3-fold honey concentration following methods outlined in the section above.

In the second stage of the screen, we further partitioned the compounds in the *Crithidia*-inhibiting flash chromatography fractions (combined after the first stage) by semi-preparative HPLC on a Waters (UK) system (600E pump, 996 PDA detector; Phenomenex Luna C18 column: 150 mm x 10 mm, 10 μm particle size). A mobile phase of methanol and water was run for 20 min at a flowrate of 4 ml/min (method: 0-1 min = isocratic gradient 10% methanol, 90% H_2O; 1-18 min = linear gradient 30% methanol, 70% H_2O to 78% methanol, 22% H_2O; 18-20 minutes = linear gradient 78% methanol, 22% H_2O to 100% methanol). We monitored UV-absorbance and collected individual peaks for further *in vitro* testing at the equivalent of 1- and 2-fold honey concentration as outlined in the section above.

To isolate the main compound from the active semi-preparative HPLC fraction, *Calluna* honey (Afon Mel, New Quay, Wales, UK) was dissolved in ultrapure water, to which brine (5.1 mol/l NaCl) and hexane were added for a final ratio of 1:4.4:1.3:2.2 (honey:H_2O:brine:hexane; by weight). This solution was shaken to form an emulsion. After separation of the emulsion, the hexane layer was collected, and shaken a second time with brine (5.1 mol/l NaCl) at a ratio of 1:2. The hexane layer was collected and dried down. We further purified the active compound using a SNAP KP-Sil 25 g cartridge on an Isolera One (Biotage, Sweden) flash purification system and a mobile phase of dichloromethane (DCM) and ethyl acetate (flow rate 10 ml/min, method: 0-1 column volume: linear gradient from 100% DCM to 92% DCM, 8% ethyl acetate; 1-8 column volumes: isocratic gradient 92% DCM, 8% ethyl acetate). The compound eluted after 17 minutes.

The isolated compound was dissolved in $CDCl_3$ and analyzed with a 400 MHz Bruker Avance nuclear magnetic resonance (NMR) spectrometer (Bruker, Billerica, MA). 1D (^{1}H, ^{13}C and DEPT) and 2D (COSY, HMBC, and HSQCDEPT) experiments were conducted to elucidate the structure and verify its purity.

We then tested the purified compound (callunene) against *C. bombi in vitro* at concentrations from 0 – 200 ppm (0 - 980 μM). We estimated *C. bombi* cell concentrations in each assay well after 7 days under a phase contrast microscope (Zeiss Photomicroscope III; Carl Zeiss AG, Germany) with a Neubauer improved counting chamber at 640-fold magnification. The IC_{50} concentration for the compound was estimated using the drc package [81] in R 3.5.1 [80], with a log-logistic 3 parameter model, and the lower limit at 0 (fct = LL.3).

Field sampling

Samples of nectar, pollen, whole flowers, and leaves of *C. vulgaris* were collected from a natural heathland fragment (Wimbledon Common, Greater London, United Kingdom) in July/August 2018. Flowers were protected with organza bags for 24 hours before collecting nectar and pollen samples. Nectar was sampled by inserting 1 μl glass microcapillaries into the flowers. *B. terrestris* workers foraging on *C. vulgaris* were caught and chilled on ice during transport to the laboratory. The honey crop, and the combined mid- and hindgut were dissected out. All samples were stored at $-20°C$ until further processing.

Chemical analysis of bumblebee and *C. vulgaris* samples

For the quantification of callunene we weighed plant, honey and bumblebee samples (Mettler Toledo Balance XS105), and extracted them in 80% methanol in a sample to solvent weight ratio of 1:9. Gut samples were macerated with plastic pestles in 1.5 mL Eppendorf tubes to facilitate extraction. Samples were vortexed at the start and end of the extraction period. After 24 hours, samples were centrifuged, and supernatants were analyzed by HPLC-MS (Velos-Pro, Thermo Fisher Scientific; Phenomenex C18 column: 150 × 3 mm, 3 μm particle size) alongside standards of pure callunene. Concentrations were estimated from the peak area of the corresponding molecular ion peak ($[M + H]^+$; m/z 205) in positive electron spray ionisation mode, using the standards of known concentrations for calibration, and accounting for dilution in 80% methanol.

Effects of *Calluna* honey extract on existing infections

We tested if extracted secondary metabolites of *Calluna* honey can reduce pre-existing infections with *C. bombi* in bumblebees. Crude ethyl acetate extracts were prepared as outlined above from *Calluna* honey (Afon Mel, New Quay, Wales, UK). Adult bumblebee workers (*Bombus terrestris audax*) sampled at random from four laboratory colonies (Biobest, Belgium) were starved for 3 hours and fed an inoculum of 15 μl containing 15000 *C. bombi* cells from the culture used in the *in vitro* experiments in 50% Apiinvert sugar syrup (Apiinvert, Südzucker, Germany). Workers were maintained individually in cages and fed *ad libitum* with 50% sugar syrup (Apiinvert) and honeybee collected, polyfloral pollen (Biobest, Belgium). After 7 days, infections were verified microscopically from fecal samples. Uninfected individuals were excluded from the experiment. The infected bumblebees were fed one of four diets: A control diet of 50% sugar syrup (Apiinvert), or a *Calluna* honey extract diet with extracts re-dissolved in 50% sugar syrup at the equivalent of 1-fold, 0.5-fold, or 0.25-fold honey concentration. Parasite loads were quantified after another 7 days on the treatment diets by collecting fecal samples from individuals in plastic vials using a glass microcapillary and counting parasite cell microscopically under a phase contrast microscope (Zeiss Photomicroscope III; Carl Zeiss AG, Germany) with a Neubauer improved counting chamber at 640-fold magnification. Cells were counted by an observer blind to the treatment group. *C. bombi* cell

concentrations were compared across treatments by fitting a linear model with the "lm" function in R 3.5.1 [80], with honey extract concentrations as continuous and colony origin as categorical fixed effects. Parasite cell concentrations were log-transformed.

Effects of *Calluna* honey extracts on infection establishment

Bumblebee workers from eight *B. terrestris audax* laboratory colonies were sampled at random and kept in individual cages. As in the preceding experiment, workers were fed either a control diet of 50% sugar syrup (Apiinvert), or a *Calluna* honey extract diet at 1-fold, 0.5-fold, or 0.25-fold honey concentration in 50% sugar syrup. After 2 days on the treatment diets, workers were starved for 3 hours, and infected with an inoculum of 15 μl containing 5000 *C. bombi* cells in 50% sugar syrup. Individuals were placed back into their cages immediately after feeding on the inoculum had been observed. After 7 days continued feeding on honey extract or control diets, we collected fecal samples from each individual and assessed parasite presence and concentrations microscopically as described above. We tested whether an increased concentration of the *Calluna* honey extract affected the infection status using a generalized linear model (GLM) with the glm function in R 3.5.1 [80]. Infection status (0/1) was treated as a binary dependent variable, with the predictors honey extract concentration as a continuous and colony origin as a categorical fixed effect (glm(Infection_status ~extract_conc + Colony, family = binomial). We calculated likelihood-ratio chisquare and p values from the GLM with the Anova function of the car package [82]. We also tested the effect of honey extract concentration on overall parasite cell concentration (log-transformed) in a linear model with the function lm in R 3.5.1 [80] (lm(log(parasite_conc+1) ~extract_conc + Colony)).

Localization of *C. bombi* in the gut

We dissected the gut out of 10 *C. bombi* infected *B. terrestris* workers that had been fed on 50% sugar water and *ad libitum* polyfloral pollen. The intact, entire gut was stretched out in a sterile 6 mm Petri dish and cut with a fine scalpel to separate the crop, anterior midgut, posterior midgut, ileum and rectum. Each segment was placed individually into a sterile 1.5 mL Eppendorf tube. Gut segments were macerated with plastic pestles (Sigma-Aldrich, USA) in 1.5 mL Eppendorf tubes with 100 μl sterile quarter-strength Ringer's solution (Thermo Fisher Scientific Oxoid, UK). *C. bombi* cell concentrations were then evaluated microscopically as described above. To examine the direct location of *C. bombi* within the gut segments, we placed gut segments into a chamber on a glass microscopy slide, using thin strips of tesa TACK adhesive putty (tesa, Milton Keynes, UK) to increase the distance of the coverslip from the slide. Chambers were filled with quarter-strength Ringer's solution (Thermo Fisher Scientific Oxoid, UK). Gut segments were filmed at 640-fold magnification under a phase contrast microscope (Zeiss Photomicroscope III; Carl Zeiss AG, Germany) with a TrueChrome Metrics microscope camera (Tucsen, China). The inside of the intact rectum was examined through the near transparent gut wall. For the ileum, gut segments had to be sliced longitudinally with a fine scalpel to allow filming of the interior.

Effects of callunene on *C. bombi* cells and infectivity

We evaluated the effect of short time exposure of *C. bombi* to callunene. We noted an apparent reduction of the flagellum to a small stump in a preliminary trial after compound exposure. We therefore exposed a culture of *C. bombi* choanomastigotes to 110 ppm of callunene (the average measured concentration in the crop of foragers) for 90 minutes and counted cells with a long functional flagellum, or a flagellum that was short or absent. *C. bombi* cells at 100 cells/μl were kept in culture medium with or without 110 ppm of callunene in 50 μl aliquots in individual wells of 96 well tissue culture plates (Eppendorf, Germany), incubated at 28°C and 3% CO_2 for 90 minutes. After incubation, cells were examined microscopically, and the first 50 cells scored as either flagellated or "deflagellated." Cells were scored by an observer blind to the treatment group. Ratios of "deflagellated" to flagellated cells from 10 replicates were compared with a Wilcoxon rank sum test in R 3.5.1 [80].

To test the effects of direct, short term callunene exposure on *C. bombi* infectivity, we first exposed *C. bombi* cells (2000 cells/μl) *in vitro* to either 200 ppm callunene (within the range of concentrations recorded in the crop of *B. terrestris* foraging on *C. vulgaris*) or as control to the same *C. bombi* liquid medium without callunene. Cells were incubated at 24°C for 60 minutes, around the average *B. terrestris* worker foraging bout time [55], to mimic a field realistic exposure of *C. bombi* parasite cells to callunene in the crop. Cell suspensions were then mixed in a ratio of 1:29 with 50% Apiinvert sugar syrup. Immediately afterward, *B. terrestris* workers selected from 3 colonies and deprived of food for 3 hours in Petri dishes were assigned randomly to each treatment and fed with 15 μl (1000 *C. bombi* cells) from either the callunene exposed or control treatment. We visually checked for complete consumption of the inoculum droplet by each worker and excluded workers that had not fed after 30 minutes. Bumblebees were then maintained in individual cages (see above) on 50% Apiinvert sugar syrup and *ad libitum* polyfloral pollen. After 7 days, we dissected out the hindgut (ileum & rectum) of each bee and measured their weights (Mettler Toledo Balance XS105). We macerated the hindguts with plastic pestles (Sigma-Aldrich, USA) in 1.5 mL Eppendorf tubes with 100 μl sterile quarter-strength Ringer's solution (Thermo Fisher Scientific Oxoid, UK). *C. bombi* cell concentrations in macerated guts were assessed microscopically as described above. We calculated *C. bombi* cells per mg hindgut taking gut weights and dilution with Ringer's solution into account. For samples without *C. bombi* cells detected in the Neubauer counting slides chambers, we examined a second 10 μl gut homogenate sample for 5 minutes in a wet mount on a regular glass microscopy slide. Individuals were considered uninfected if no *C. bombi* cells were detected in either case. We used a generalized linear mixed model (GLM) with the glm function in R 3.5.1 [80] to test for significant differences in infection rate after callunene exposure. Infection status (0/1) was treated as a binary dependent variable, with the predictors Treatment (callunene/control) as a binary and colony origin as categorial fixed effect (glm(Infected ~Treatment + Colony), family = binomial)). We calculated likelihood-ratio chisquare and p values from the GLM with the Anova function of the car package [82]. We also tested the effect of

callunene pre-infection treatment on the overall parasite cell concentration in the hindgut (log-transformed) in a linear model with the function lm in R 3.5.1 [80] (lm(log(cells.mg+1) ∼Treatment + Colony)).

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed in R 3.5.1 [80]. The IC_{50} concentration for callunene was estimated using the drc R package [81], with a log-logistic 3 parameter model, and the lower limit at 0 (fct = LL.3). We tested for significant inhibition of all extracts with an ANOVA and a subsequent post hoc Dunnett's test, comparing each treatment to the medium with 1% methanol control, using the multicomp package [79] in R 3.5.1 [80]. Ratios of ''deflagellated'' to flagellated cells from 10 replicates were compared with a Wilcoxon rank sum test. *C. bombi* cell concentrations in the experiment feeding infected *B. terrestris* workers with heather honey extracts of different concentrations were compared across treatments in a linear model with the ''lm'' function in R 3.5.1 [80] with honey extract concentration as a continuous and colony origin as a categorical fixed effect variable. Parasite cell concentrations were log-transformed. To test whether feeding on increased concentration of the *Calluna* honey extract affected the infection rate of *B. terrestris* workers (prophylactically), we used a generalized linear model (GLM) with the glm function in R 3.5.1 [80]. Infection status (0/1) was treated as a binary dependent variable, with the predictors honey extract concentration as a continuous and colony origin categorical fixed effects (glm(Infection_status ∼extract_conc + Colony), family = binomial). We calculated likelihood-ratio chisquare and p values from the GLM with the Anova function of the car package [82]. We also tested the effect of honey extract concentration on overall parasite cell concentration (log-transformed) in a linear model with the function lm in R 3.5.1 [80] (lm(log(parasite_conc+1) ∼extract_conc + Colony)).

DATA AND CODE AVAILABILITY

The datasets generated during this study are available on Mendeley Data (https://doi.org/10.17632/866cffncvf.1).