Flagellum removal by a nectar metabolite inhibits infectivity of a bumblebee parasite

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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

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.

Summary

Plant secondary metabolites can act as natural "medicines" for animals against parasites [1, 2, 3]. Some nectar metabolites, for example, reduce parasite infections in bees [4, 5, 6, 7]. Understanding these interactions is urgent, as bees provide critical pollination services [8, 9, 10, 11] but are threatened by interacting stressors including diseases [11, 12, 13, 14, 15, 16, 17]. Declining plant diversity through anthropogenic landscape change [18, 19, 20, 21] could reduce the availability of medicinal nectar plants for pollinators, exacerbating their decline [22]. Existing studies are, however, limited by (i) a lack of mechanistic insights into how plant metabolites affect pollinator diseases, and (ii) 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 common bumblebee pathogen *Crithidia bombi* (Trypanosomatidae) [16, 23, 24, 25, 26]. The most bioactive species was heather (*Calluna vulgaris*), the second most productive UK nectar plant [20]. We identified 4-(3-oxobut-1-enylidene)-3,5,5-trimethylcyclohex-2-en-1-one (callunene) from heather nectar as 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 *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 plant secondary metabolites can disrupt parasite flagellum attachment, revealing a mechanism behind their prophylactic effects. The decline of heathlands [27, 28, 29, 30] reduces the availability of

natural bee “medicine” and could exacerbate the contribution of diseases to pollinator declines.

Keywords: pharmacognosy, phytochemistry, host-parasite ecology, parasitology, entomology, pollinator, bumblebee, flagellum, honey, drug discovery

Results & 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 [31, 32]. Some of these metabolites exhibit antimicrobial activity and may have medicinal benefits for bees against their diseases [6, 22, 33, 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 [24] that reduces bumblebee fitness by strongly decreasing colony founding success in queens [26], and through decreased starvation tolerance in workers [25]. The parasite transmits between bumblebees through ingestion of cells shed in the faeces of infected hosts, and establishes infections in the gut of the host [25].

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. [20]: 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 (Fig. 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, while for the remaining monofloral honey extracts parasite growth was within the range of the controls (Fig. 1A). Here we focussed further experiments on *C. vulgaris*, as the plant with the most potent inhibitory honey extract and the second largest contributor to nectar provision in the UK [20]. We combined chromatographic fractions with significant inhibitory activity from the first stage of our bioactivity-directed-fractionation of the *Calluna* honey extract (Suppl. Fig. 1A) 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* (Suppl. Fig. 1B). 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 (Fig. 1B, Suppl. Fig. 2, Suppl. Table 1). We assign the trivial name callunene to this compound. The IC_{50} of callunene against *C. bombi* was estimated at 23 ppm (113 μM) (Fig. 1B), when testing callunene concentrations from 0-200 ppm (0 - 980 μM).

We detected callunene in the nectar of wild *C. vulgaris* plants at concentrations comparable to those found in honey extracts (Table 1, Suppl. Fig. 3). 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, Suppl. Fig. 3). 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 focussed 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 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, 43, 44]), 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 (GLM, $\chi^2 = 4.3$, $p = 0.037$) (Fig. 2A), with a significant colony effect on infection outcome (GLM, $\chi^2 = 14.7$, $p = 0.04$). Higher extract concentrations also resulted in lower overall infection intensities ($F(1,105) = 8.2$, $p = 0.0052$) (Fig. 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

(Fig. 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, Suppl. Fig. 3). In contrast, the midgut and hindgut had either very low or undetectable levels, mirroring our findings in field-collected animals (Table 1, Suppl. Fig. 3) and suggesting the compounds had been metabolised. 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 & rectum) of the host, with the highest concentration in the ileum (Suppl. Fig. 4). *C. bombi* would therefore be exposed to high concentrations of callunene only during passage though 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 like 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, 34, 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 *C. bombi* cells. Short time exposure (90 minutes) *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, $W = 100$, $p = 0.00018$) (Fig. 3A, Suppl. Video 1). We note that the doubling time of *C. bombi* has been estimated at 10-16 hours [54], and therefore our exposure

timeframe excludes inhibition of flagellum formation in new cells as an explanation for the observed absence of the flagellum in treated cells. Suppl. Video 1 furthermore shows the gradual loss of a functional flagellum of an individual *C. bombi* cell within under 20 minutes 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 (Suppl. Video 2). 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 minutes (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, $\chi^2 = 5.6$, $p = 0.018$), and an overall lower infection load ($F(1,54) = 7.4$, $p = 0.0089$) after 7 days (Fig. 3B), with no significant impact of colony identity on infection rate (GLM, $\chi^2 = 0.4$, $p = 0.81$) 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 [34], 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 if 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 like 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 [20, 61, 62, 63, 64, 65]. In a study estimating the amount of nectar sugar produced by flowering plants in the United Kingdom, *C. vulgaris* ranked 2nd place by total yearly amount [20]. 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 if their presence reduces infections at the landscape level. Alarmingly, heathlands are declining globally due to land use changes and eutrophication [27, 28, 29, 30]. 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 [21, 67, 68]. In the 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, 72, 73, 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.

Conclusion

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* (Fig. 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.

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Author contributions

Conceptualization, H.K., J.W., M.J.F.B. and P.C.S.; Methodology, HK and JW; Formal Analysis, H.K., J.W., and MKL; Investigation, H.K., J.W., and MKL; Writing - Original Draft, HK; Writing - Review & Editing HK, JW, MJFB and PCS; Funding Acquisition, P.C.S. and M.J.F.B.

Declaration of interests

The authors declare no conflict of interests.

Figure titles & legends

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 bar). Arrow marks *Calluna* heather extract; "Medium Control": growth in pure *C. bombi* medium; "Medium+MeOH": growth in *C. bombi* medium with 1% methanol. Asterisks behind 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 growth under different callunene concentrations (6 replicates per treatment). To the right: Structure of callunene (top) and *B. terrestris* foraging on *Calluna vulgaris* (bottom).

Figure 2. Heather honey extract prevents *Crithidia bombi* infections in bumblebees, but does not cure existing ones
A: *C. bombi* infection load in *B. terrestris* workers that fed on different heather honey extract concentration (1 = 1-fold honey concentration) two days prior to infection and during the 7 days after infection. *C. bombi* cell concentrations were assessed in faecal samples 7 days after infection.
B: *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 extracts concentration (1 = 1-fold honey concentration). *C. bombi* cell concentrations were assessed in faecal samples after 7 days on the heather honey extract diet.

Figure 3. Callunene removes the flagellum, *Crithidia* loses infectivity
A: Percentage of *C. bombi* cells with flagellum reduced to small stump or entirely absent (see left inserted picture and Suppl. Video 1) after 90 minutes 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: *C. bombi* concentrations per µg of hindgut weight 7 days after infection with cells either treated for 60 minutes 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 half:** 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 half:** 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.

Tables

Table 1. Concentrations (ppm) of callunene in *C. vulgaris* plant samples, and *B. terrestris* wild and laboratory worker gut segments. Laboratory workers had fed on 1-fold heather honey extract for 7 days at the time of dissection.

Sample	Average	Range	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- & 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

STAR★Methods

Contact for Reagent and Resource Sharing

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).

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 Fig. 2A, 4 colonies for the experiment in Fig. 2B, 3 colonies for the experiment in Fig. 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 grams of each honey was first dissolved in 10 grams of ultrapure water (Milli-Q; Sigma, St. Louis, MO) and shaken with 10 grams 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 seconds. 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 grams 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 25g 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 analysed with a 400 MHz Bruker Avance nuclear magnetic resonance (NMR) spectrometer (Bruker, Billerica, MA). 1D (^1H, ^{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 analysed by HPLC-MS (Velos-Pro, Thermo Fisher Scientific; Phenomenex C18 column: 150 x 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 honey bee collected, polyfloral pollen (Biobest, Belgium). After 7 days, infections were verified microscopically from faecal 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 faecal 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 faecal 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 cover slip 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 afterwards, *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 Software Availability

The datasets generated during this study will be made publicly available on figshare before publication.

Key Resources Table

See separate file.

Supplemental item titles

Suppl. Figure 1. *C. bombi in vitro* growth measured as change in optical density (at 620 nm) when treated with:
A) the 10 flash chromatography fractions of the *Calluna vulgaris* ethyl acetate extract, compared to controls and total extract. Activity measured at 3.3X honey concentration;
B) each of the nine peaks derived from semi-preparative HPLC of active fractions (Nos. 5,6,7) in Suppl. Fig. 1A. Activity measured at 1X and 2X honey concentration. Peak 8: callunene.
Asterisks behind treatments indicate significantly lower increase in OD compared to Medium+MeOH control, i.e., significant growth inhibition by the fractions (Dunnett's test, ***: $p < 0.001$, **: $p < 0.01$.

Suppl. Figure 2. NMR spectra of callunene isolated from *Calluna* honey.
A) 400 Hz ^{1}H NMR spectrum; **B)** 100 Hz ^{13}C NMR spectrum; **C)** COSY NMR spectrum; **D)** HMBC NMR spectrum; **E)** 1D ^{13}C DEPT NMR spectrum; **F)** HSQCDEPT NMR spectrum; **G)** Structure of callunene with numbered carbon atoms, related to structure of callunene in Figure 1 and chemical shifts in Supplementary Table 1.

Suppl. Figure 3. Concentration (ppm) of callunene in *C. vulgaris* plant parts, and gut segments of wild or experimental laboratory *B. terrestris* workers. (MG = midgut, HG = hindgut, exp. = experimental, wild = collected foraging on *C. vulgaris*). Related to Figure 1 & Table 1.

Suppl. Figure 4. Concentration of *C. bombi* cells in gut segments of infected *B. terrestris* workers macerated in Ringer's solution. (AMG = anterior midgut; PMG = posterior midgut). Related to Figure 3C.

Supplementary Table 1: ^{13}C and ^{1}H NMR chemical shifts (in $CDCl_3$) plus HMBC and COSY analyses for 4-(3-oxobut-1-enylidene)-3,5,5-trimethylcyclohex-2-en-1-one (callunene). ^{13}C NMR shifts for callunene from Tan et al. [36] for comparison. See Suppl. Figure 9 for chemical structure. Note that δ ^{13}C values for carbon atoms 8, 9, and 4' are incorrectly assigned in Tan et al. [36] and are here corrected based on 2D NMR spectra. Related to callunene structure in Figure 1.

Supplementary Video 1: Callunene induces flagellum removal. *C. bombi* cells exposed *in vitro* to callunene; first sequence: *C. bombi* unexposed control t = 90 min, motile cells with long flagellum; second sequence: *C. bombi* exposed to 110 ppm callunene, t = 90 min, cells without functional flagellum; third sequence: consecutive movies of the same *C. bombi* cell, during exposure to 200 ppm callunene in a wet mount on a microscopic slide at t = 10 minutes, t = 13.5 minutes & t = 17.5 minutes showing gradual loss of flagellum and motility.

Supplementary Video 2: *C. bombi* attaches with its flagellum to the ileum. First sequence: *C. bombi* cells attach along the epithelium of the ileum with the flagellum, cell body moving in the lumen; second sequence: close up of *C. bombi* cells attached to ileum epithelium with its flagellum; third sequence: *C. bombi* cells move and float freely in the lumen of the rectum (filmed through transparent wall of an intact rectum).

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