RESEARCH ARTICLE

Weak and contradictory effects of self-medication with nectar nicotine by parasitized bumblebees [v1; ref status: indexed, http://f1000r.es/56l]

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Abstract

The presence of antimicrobial secondary metabolites in nectar suggests that pollinators, which are threatened globally by emergent disease, may benefit from the consumption of nectars rich in these metabolites. We tested whether nicotine, a nectar secondary metabolite common in *Solenaceae* and *Tilia* species, is used by parasitized bumblebees as a source of self-medication, using a series of toxicological, microbiological and behavioural experiments. Caged bees infected with *Crithidia bombi* [TI1] had a slight preference for sucrose solution laced with the alkaloid and behavioural tests showed that the parasite infection induced an increased consumption of nicotine during foraging activity. When ingested, nicotine delayed the progression of a gut infection in bumblebees by a few days, but dietary nicotine did not clear the infection, and after 10 days the parasite load approached that of control bees. Moreover, when pathogens were exposed to the alkaloid prior to host ingestion the protozoan's viability was not directly affected, suggesting that anti-parasite effects were relatively weak. Nicotine consumption in a single dose did not impose any cost even in food-stressed bees (starved) but the alkaloid had detrimental effects on healthy bees if consistently consumed for weeks. These toxic effects disappeared in infected bees suggesting that detoxification costs might have been counterbalanced by the advantages in slowing the progression of the infection. Nonetheless we did not find a benefit of nicotine consumption in terms of life expectancy of infected bees, making these findings difficult to interpret. Our results indicate that caution is warranted in interpreting impacts of plant metabolites on insect parasites and suggest that the conditions under which nicotine consumption provides benefits to either bees or plants remain to be identified. The contention that secondary metabolites in nectar may be under selection from pollinators, or used by plants to enhance their own reproductive success, remains to be confirmed.

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Introduction

Parasites can have a dramatic impact on their hosts, and consequently provide a powerful selective force for host defence mechanisms. Molecular mechanisms (e.g. the innate and adaptive immune system) are traditionally considered the major anti-parasite defences in the animal kingdom. However, hosts can rely on a range of alternative defence mechanisms, such as morphological barriers (St Leger, 1991), changes in life-history traits (Michalakis, 2009), symbiont-mediated defences (Oliver *et al.,* 2010) and altered behaviours (de Roode & Lefèvre, 2012; Moore, 2002).

Behavioural immunity is an important modality of defence against diseases (de Roode & Lefèvre, 2012), and medication behaviour is a key immune mechanism in some animals (Clayton & Wolfe, 1993; de Roode *et al.*, 2013). Medication behaviour has been defined as the use of anti-pathogenic substances found in the environment or produced by other species or individuals (Lozano, 1998). In therapeutic medication, sick individuals alter their behaviour to medicate themselves in response to parasites (Singer *et al.*, 2009), while prophylaxis is displayed by healthy individuals in response to parasite risk rather than infection (Castella *et al.*, 2008). For example, wood ants, bees and wasps behave prophylactically to incorporate conifer resin, propolis, or venom containing antimicrobial compounds into their nest, which inhibits the growth of bacteria and fungi (Baracchi & Turillazzi, 2010; Baracchi *et al.*, 2011; Chapuisat *et al.*, 2007; Castella *et al.*, 2008; Simone *et al.*, 2009), while, from a therapeutic perspective, ants apply antimicrobial venomous secretion to the cuticle of contaminated larvae to medicate their brood (Tragust *et al.*, 2013). So far, most evidence for animal self-medication comes from the consumption of curative plants by vertebrates (Rodriguez & Wrangham, 1993). For example, chimpanzees, *Pan troglodytes,* alter their foraging to include medicinal substances (particular plant species) in their diets to cure helminth infections (Wrangham, 1995). Plants are good candidates for prophylactic or therapeutic foods as they often contain metabolites that display a wide range of biological activities (Cowan, 1999) which were originally evolved to combat herbivores or plant-parasites (Hadacek, 2002). This preferential ingestion of "non-nutritive" food and chemicals to self-medicate is known as pharmacophagy or zoopharmacognosy. Despite numerous studies investigating feeding plasticity with respect to plant nutrients and medicinal metabolites (reviewed in Mooney & Agrawal, 2008), investigations of potential pharmacophagy are rare, especially in insects. Exceptions concern self-medication behaviour described in two species of woolly bear caterpillars, which increase their preference for pyrrolizidine alkaloids or iridoid glycosides when parasitized, improving their chances of surviving parasitoid infection (Bernays & Singer, 2005; Singer *et al.*, 2009; Smilanich *et al.*, 2011). Similarly, wasp-infected fruit fly larvae preferentially consumed high-ethanol fly food as a medicine against their parasitoid wasp larvae, again increasing their survival (Milan *et al.*, 2012), while no evidence for self-medication to nematode parasitism has been found in the fly *Drosophila putrida* (Debban & Dyer, 2013). Trans-generational medication, but not self-medication, has been described in the monarch butterfly (Lefevre *et al.*, 2010) and self-medication has been hypothesized for honeybees that increase plant resin collection in response to a fungal infection (Simone-Finstrom & Spivak, 2012).

Animal societies arguably face the most intense pressure from pathogens. This pressure is enhanced in insect societies due to a suite of traits, including the high number of individuals living in high densities, relatively low genetic variability, and the relatively stable, high levels of humidity and temperatures of their nests (Schmid-Hempel, 1998). In addition, social pollinators, such as bumblebees and honeybees, are often exposed to an increased risk of infection via flowers (reviewed in McArt *et al.*, 2014), which represent a shared "public place" where homo- and hetero-colonial conspecifics and other heterospecific pollinators feed repeatedly every day. Given the potential importance of parasites and disease in driving declines of managed honeybees (de Miranda & Genersch, 2010; Rosenkranz *et al.*, 2010) and wild bumblebees (Cameron *et al.*, 2011; Fürst *et al.*, 2014; Schmid-Hempel *et al.*, 2014), understanding the potential relevance of pharmacophagy to social pollinators may be a key to understanding and managing these declines.

Here we use an important natural and managed pollinator, the bumblebee *Bombus terrestris*, and its parasite *Crithidia bombi* to investigate the potential for pharmacophagy in social pollinators. *C. bombi*, a trypanosome gut parasite, is the most prevalent parasite of bumblebees (Shykoff & Schmid-Hempel, 1991). The parasite, transmitted either vertically or horizontally (Durrer & Schmid-Hempel, 1994; Otterstatter & Thomson, 2007), infects adults *per os,* and two-three days post infection, infective cells are released through the faeces of bees (Schmid-Hempel & Schmid-Hempel, 1993). Queens infected by *C. bombi* have a reduced success in colony founding (Brown *et al.*, 2003), and produce fewer reproductive offspring (Brown *et al.*, 2003), while infected workers experience a higher mortality rate under stressful conditions (Brown *et al.*, 2000). Moreover, infection impairs foraging success and learning abilities, inducing additional costs to the colony (Alghamdi *et al.*, 2008; Gegear *et al.*, 2006). Recent research (Manson *et al.*, 2010; Richardson *et al.*, 2015) has shown that several secondary metabolites such as alkaloids (including nicotine) and glycosides, reduce the *C. bombi* load after consumption by the bumblebee species *Bombus impatiens*, suggesting that these pollinators might exploit nectar toxins or other metabolites to self-medicate.

To test whether bumblebees are able to self-medicate using naturally occurring nectar secondary metabolites we conducted a series of toxicological, microbiological and behavioural experiments using a different species of *Bombus* (*B. terrestris*) and *C. bombi* as models and nicotine as a natural nectar alkaloid. Nicotine is encountered by pollinators at variable concentrations between 0.1 ng/µl and 3 ng/µl in floral nectar of *Nicotiana* species (native of South America and naturalised worldwide by humans) and *Tilia* species (native throughout most of the temperate Northern Hemisphere) (Detzel & Wink, 1993; Naef *et al.*, 2004; Tadmor-Melamed *et al.*, 2004).

Methods

Insects and pathogens

All experiments were performed with worker bumblebees (*B. terrestris*) obtained from a continuous rearing program (provided by Koppert B.V., The Netherlands) and conducted under standardized laboratory conditions. The insects were provided *ad libitum* with commercial pollen (provided by Koppert B.V., The Netherlands)

and 30% sucrose solution as protein source and energy respectively. The parasites (the protozoan flagellates *C. bombi*) used for the experimental infections were taken from several naturally infected colonies that we started in the laboratory from infected queens.

Infection experiments

To determine whether the nectar alkaloid nicotine influences the severity of *C. bombi* infections in bumblebees, we designed two experiments following (Manson *et al.*, 2010). In the "Continuous Exposure" test, bees were first inoculated with *C. bombi* and then fed on a daily diet of a nicotine solution or sucrose solution (Control), simulating the continual ingestion of nectar constituents by an infected foraging bee. In the "Delayed Exposure" test, we first exposed directly *C. bombi* cells to nicotine or control solutions for two hours before inoculating bees, and then we fed them on a sucrose-only solution. We subsequently compared the parasite load in inoculated bumblebees.

A mixture of different parasite strains was prepared by collecting faeces from 30 workers from three infected colonies. The faeces were mixed for one minute with a vortex mixer and the *C. bombi* cocktail was allowed to settle at room temperature for two hours, after which the supernatant was removed and mixed thoroughly. Cell counts were made using a haemocytometer. The faeces were then mixed with sugar water to produce an inoculum concentration of 2,000 parasite cells/µl. Prior to inoculation, bees were deprived of all food for two hours to facilitate infection. Bees derived from two different healthy colonies were screened to make sure that the bees were parasite-free. Each bee was then presented with a 10 µl drop of inoculum and observed until the inoculum was drunk. Thus, each bee ingested a total of 20,000 parasite cells. This dose falls within the range of *C. bombi* cells present in faeces from infected workers (Logan *et al.*, 2005), and therefore simulates cells available for transmission to healthy bees.

Post inoculation, in the "Continuous Exposure" test, bees from three colonies were kept individually in Petri dishes and received either a 0.5 ml solution of 2.5 ng/µl nicotine (nectar concentration in the natural range of this alkaloid) in 30% sucrose (Experimental bees, $n = 20$) or 0.5 ml of 30% sucrose only (Control bees, $n = 20$) along with a 1g pollen lump daily for 10 days. In the "Delayed Exposure" test the *C. bombi* inoculum was exposed to nicotine in the dark for two hours prior to host ingestion, simulating direct exposure of the pathogen to nectar in a flower. C. *bombi* cells were placed in a solution of 2.5 ng/µl nicotine in 30% sucrose (Experimental treatment), and in a solution of 30% sucrose only (Control treatment). Two hours later 20 Experimental bees and 20 Control bees were inoculated (for inoculum preparation see above). The treatment simulates the period between the deposition of *Crithidia* cells by infected bees and the next flower visit by a naïve bee. Post inoculation, bees of both groups were kept in individual Petri dishes and given 0.5 ml of 30% sucrose solution and a fresh pollen lump daily.

In both experiments, the infection levels were checked at day 7 and day 10 post inoculation (the period of time in which pathogen load is saturated (Schmid-Hempel & Schmid-Hempel, 1993)). Each bee was removed from its Petri dish and put into a small glass tube until it defecated. In cases when no or too little rectal fluid was obtained, the procedure was repeated for that bee a few hours later. Faeces were transferred to a haemocytometer to count the number of parasite cells.

Laboratory toxicity bioassays

In order to determine the impact of nicotine consumption on bumblebee survival and any possible interactive effects of dietary toxin consumption and physiological stress (for which we used starvation, as *Crithidia* has its biggest detrimental impacts on starved bees (Brown *et al.*, 2000)), we conducted a series of experiments in which we exposed bumblebees to artificial nectars enriched with nicotine maintained either starved or provided with *ad libitum* food. "Starved bees" were moved individually from their nest into Petri dishes, starved for two hours and fed either with *ad libitum* 30% sucrose solution food for 30 minutes (Starved, Control) or 2.5 ng/µl nicotine in 30% sucrose (Starved, Nicotine). Survival censuses were conducted every hour until all bees were dead. "*Ad libitum* food bees" were kept individually in Petri dishes, and given 0.5 ml of 30% sucrose solution and a fresh pollen lump daily (Control *ad libitum* food), 2.5 ng/µl nicotine in 30% sucrose solution and a fresh pollen lump daily (Nicotine *ad libitum* food), 2.5 ng/µl nicotine in 30% sucrose solution on day 0 and 0.5 ml of 30% sucrose solution and a fresh pollen lump daily (Nicotine-once *ad libitum* food). Survival censuses were conducted daily until all bees had died. For each of the five treatments we chose bees from three different young healthy colonies and we randomised bees across treatment groups. Each treatment group was composed of 60 bees (20 bees per colony). Comparisons of the survival parameters of bumblebees in all treatments allowed us to evaluate the effect of nicotine, starvation, and colony membership on survival. Dead bees were immediately weighed using a microscale (Navigator N30330, Ohaus, Pine Brook, USA).

Trade-off between detrimental and beneficial effects of nicotine

In order to evaluate whether infected bees benefit from the consumption of nicotine in terms of survival and/or parasite load we conducted two additional experiments in which infected bumblebees received artificial nectars enriched with nicotine or not and were maintained either starved (three groups of 30 bees, 10 bees from three different colonies, 90 bees in total) or provided with *ad libitum* food (three groups of 45 bees, 15 bees from three different colonies, 135 bees in total). In both experiments the three groups of bees were inoculated with *C. bombi* as described above and individually kept in Petri dishes under three types of diet (each diet consisted of two solutions dispensed by two different Eppendorf tubes): Control Group: 30% sucrose only in both dispensers (Suc-Suc group); Exp. Group 1: 2.5 ng/µl nicotine in 30% sucrose in both dispensers (Nic-Nic group); Exp. Group 2: 30% sucrose only in one dispenser and 2.5 ng/µl nicotine in 30% sucrose in the other one (Suc-Nic group). "Starved bees" were fed for 12 days and then starved until all bees were dead. The infection levels were checked at day 7 and day 10 post inoculation. Survival censuses were conducted every hour (starved bees) and every day (*ad libitum* food bees) until all bees were dead. At the end of the experiment we quantified total consumption of artificial nectars in each dispenser for each bee. Comparison of the survival parameters of bumblebees in all treatments allowed us to evaluate the effect of nicotine and starvation on survival.

Behavioural test

For testing, each bee colony was housed in a wooden nest box (28 × 20 × 11 cm) connected to a wooden flight arena with a transparent, UV-transmitting Plexiglas lid (120 × 100 × 35 cm), by means of a transparent Plexiglas tube. Shutters along the length of this tube enabled control of the traffic of bees between nest boxes and flight arena (Chittka, 1998). Each bumblebee was individually marked with a coloured numbered disk.

Bees were pre-trained to forage on 12 square transparent plastic flowers of 24 × 24 mm (Perspex® Neutral) organized in two patches equidistant from the entrance of the nest. Plastic chips were placed on vertical transparent glass cylinders to raise them above the green floor of the flight arena. During the pre-training all flowers were rewarding with a 15 µl droplet of 30% sucrose solution, placed in a well in the centre of the flower (Raine & Chittka, 2008). This provided bees with an equal chance to associate both these patches (left and right) with reward during the pre-training period. Bees were allowed to forage freely on these flowers which were refilled as soon as the bees moved on a different artificial flower. In this way bees never experienced an empty flower with the exception of the last visited one. The number of foraging trips (bouts) made in the flight arena by each bee was observed to ensure only strongly motivated foragers visiting both patches (bees that did at least five consecutive foraging bouts) were selected for the experiment (Raine *et al.*, 2006).

After pre-training, the preference of both healthy and infected pre-trained bees was tested for blue plastic flowers (Perspex® 727) containing nicotine (one patch reward: 2.5 ng/µl nicotine in 30% sucrose solution; one patch reward: only 30% sucrose solution). Each bee ($n = 31$ infected bees; $n = 28$ healthy bees) was tested individually and one hundred consecutive choices were recorded after the first bout was initiated. Bees were regarded as choosing a flower when they landed and fed from it. Bees approaching or just briefly landing on a flower were not considered as choosing that flower. As in the pre-training, flowers were refilled after the bee moved to a different one so that bees never experienced an empty flower with the exception of the last visited one. Flowers were washed between subsequent bees in order to remove possible scent marks (Saleh & Chittka, 2006). The patch formed by nicotine-containing flowers was swapped from left to right for half the bees of each group (healthy and infected bees). Controlled illumination was provided by high frequency fluorescent lighting [(TMS 24F) lamp with HF-B 236 TLD (4.3 Khz) ballasts, Phillips, Netherlands fitted with Activa daylight fluorescent tubes, Osram] which simulated natural daylight (Dyer & Chittka, 2004). At the end of the experiment all the bees were sacrificed and the concentration of *C. bombi* in their hind gut was determined (see above).

Statistical analysis

In the infection experiments 10 out of 80 of bees died before day 10 for unknown causes. In total, we analysed the infection intensities of 40 (day 7) and 36 (day 10) bees in the "Continuous Exposure" experiment, and 37 (day 7) and 34 (day 10) bees in the "Delayed Exposure" experiment. To compare differences in parasite load between control and experimental bees at day 7 and day 10 post inoculation in both experiments we used a generalized linear mixed model (GLMM), with pathogen counts as the within-subject variable and *C. bombi* exposure to nicotine, time (day 7 and day 10), colony of origin, and bee body weight as explanatory factors. As the data were not normally distributed and homogeneity of variances and sphericity could not be assumed in several cases, we performed corrections according to Huynh-Feldt epsilon (Field, 2009). For the statistical evaluations in the survival experiments, we used the classical survival parameters including the survival distribution, percent survival at the end of the census period, median survival time (LT50), and the hazard ratio of death, using the Cox Proportional Regression analysis to generate the Wald Statistic. The hazard function characterized the instantaneous rate of death at a particular time while controlling for the effect of the other variables on survival. The following variables were entered in the regression model: colony of origin, body weight, nicotine treatment. The survival distributions for all treatments were computed and analysed with the Breslow Statistic (Mantel–Cox Test). For the behavioural experiment, a T test was used to examine differences between preferences for nicotine-rich nectar and control nectar in healthy and infected bees. Spearman rank correlation tests were used to correlate parasite load and nicotine preference. All statistical analyses were done on SPSS 13® for Windows.

Results

Infection experiments

In the "Continuous Exposure" test, a diet enriched with nicotine reduced the intensity of *C. bombi* infections in bumble bees (Dataset 1). GLMM analysis revealed significant main effects of nicotine and time since inoculation on infection intensity, but not colony of origin or bee body weight (Table 1). At both 7 days and 10 days post-inoculation, bees exposed to nicotine had infections that were, on average, 1.11 and 0.56 times respectively less intense than control bees (t test, day 7: $n = 20\text{-}20$, $t = 5.2$, df = 38, $P < 0.001$; day 10 $n = 18\text{-}18$, $t = 3.47$, $df = 34$, $P = 0.001$; Figure 1). Infection intensities increased significantly from day 7 to day 10, independently of nicotine treatment (no-significant Nicotine and Colony x Time effect; Table 1).

Table 1. "Continuous Exposure" test: results from GLMM analysis of *C. bombi* population dynamics in bumblebees.

Factor	*F-value*	*Df*	*P-value*
Nicotine	35.3	1,61	0.001
Time	16.2	1,61	0.001
Bee body weight	1.07	1,61	0.3
Colony	0.46	1,65	0.8
Interactions	-	-	N.S.

In the "Delayed Exposure" test, exposing *C. bombi* to nicotine for two hours before inoculation had no effect on parasite load (Table 2) (Dataset 1). At 7 days and 10 days post-inoculation, bees exposed to nicotine had infections that on average were as intense as those of control bees (t test, day 7: n = 19-18, t = 0.16, df = 35, P = 0.87; day 10: n = 17-17, t = -0.69, df = 32, P = 0.5; Figure 2). Infection intensities increased significantly from day 7 to day 10, independently of nicotine treatment (there was no-significant Nicotine x Time and Colony x Time effects; Table 2). Taken together, these findings prove the antimicrobial activity of nicotine against the pathogen when ingested by bumblebees, but also indicate that when pathogens are exposed to the alkaloid prior to host ingestion the protozoan's viability is not directly affected.

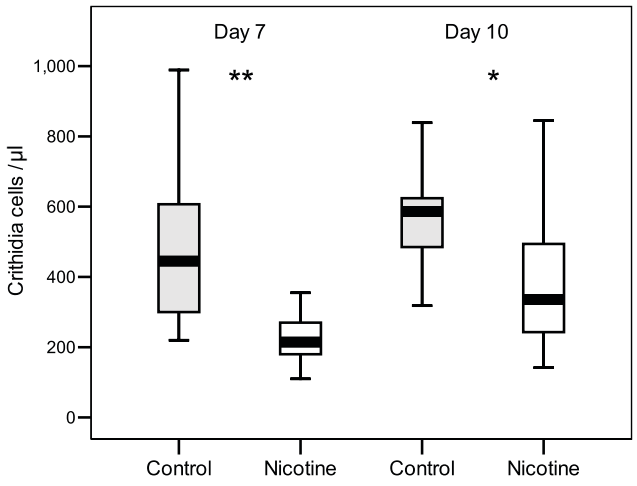

Figure 1. Intensity of *C. bombi* infections in bumblebees received either a nicotine diet (Experimental bees, *n* = 20) or a sucrose only diet (Control bees, *n* = 20). Faeces were checked after 7 days and 10 days post inoculation. Box plots show medians, 25th and 75th percentiles (**P < 0.001; *P = 0.001).

Dataset 1. Infection experiments

http://dx.doi.org/10.5256/f1000research.6262.d44610

Effect of nicotine on parasite load in infected bumblebees.

Table 2. Delayed Exposure test: results from the GLMM analysis of *C. bombi* population dynamics in bumblebees.

Factor	*F-value*	*Df*	*P-value*
Nicotine	0.02	1,62	0.8
Time	27.1	1,60	0.001
Bee body weight	0.52	1,62	0.4
Colony	2.9	1,62	0.1
Interactions	-	-	N.S.

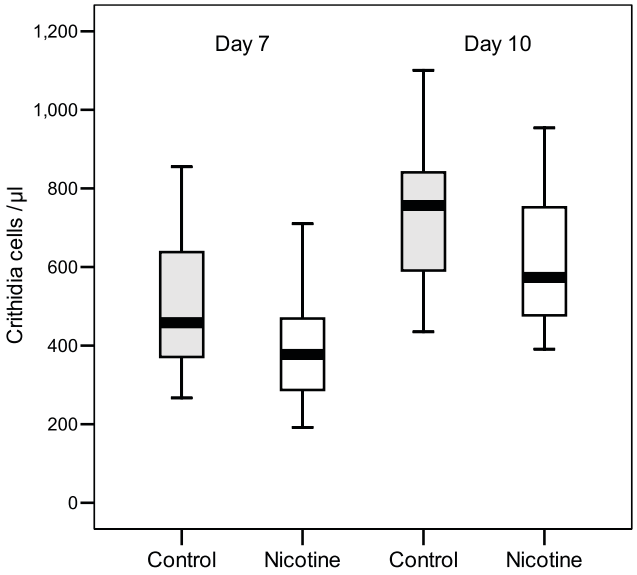

Figure 2. Intensity of *C. bombi* infections in bumblebees inoculated with pathogens previously exposed to nicotine for two hours (Experimental bees, *n* = 20) or to a control sucrose diet (Control bees, *n* = 20). Faeces were checked after 7 days and 10 days post inoculation. Box plots show medians, 25th and 75th percentiles (P = N.S.).

Laboratory toxicity bioassays

In the "Starved" test, statistical evaluation of the survivorship of control and experimental bumblebees revealed that a nicotine diet was not a significant predictor of mortality (Log-rank Mantel Cox test χ^2 = 0.21, df = 1, P = 0.88; Figure 3A) (Dataset 2). Furthermore no effect of colony of origin and bee body weight on mortality was found (GLM, treatments: F = 1.1, df = 1, P = 0.29; Colony F = 0.46, df = 2, P = 0.63; body weight: F = 0.19, df = 1, P = 0.66). The median lethal time (LT50) for the two groups did not differ (control LT50: 39 hours, exp. bees LT50 = 37 hours).

In the "*ad libitum* food" test a Log-rank Mantel Cox test showed that a daily diet including nicotine was a significant predictor of mortality (χ^2 = 11.56, df = 2, n = 180, P = 0.003; Figure 3B) (Dataset 2). Pairwise statistical comparisons revealed that bees fed consistently with nicotine had significantly lower survivorship than 'Nicotine-once' and 'Control bumblebees' (P = 0.001), while the latter two experimental groups did not differ (P = 0.86). LT50 of bees fed daily with nicotine was 39 days while 'Nicotine-once' bumblebees and control bees had a LT50 of 44 and 43 days respectively. Colony of origin and body weight did not affect bee mortality (GLM, Colony: F = 0.35, df = 2, P = 0.71; body weight: F = 1.90, df = 1, P = 0.16), but we found a significant interaction between body weight and treatment (larger bees were less susceptible to nicotine, GLM, F = 5.12, df = 1, P = 0.025). Taken together, these findings indicate that nicotine has some detrimental effects on healthy bumblebees if consistently consumed for weeks but also that these effects are possibly quite weak.

Dataset 2. Laboratory toxicity bioassays

http://dx.doi.org/10.5256/f1000research.6262.d44612

Effect of nicotine on healthy bee survival.

Trade-off between detrimental and beneficial effects of nicotine

In both "*ad libitum* food bees" and "starved bees" tests, a nicotine diet was not a significant predictor of survival (Log-rank Mantel Cox test: "*ad libitum* food bees": n = 135, Nic-Nic vs Nic-Suc $\chi^2 = 0.3$, $P = 0.6$; Nic-Nic vs Suc-Suc $\chi^2 = 0.01$, $P = 0.9$; Nic-Suc vs Suc-Suc $\chi^2 = 0.7$, $P = 0.4$; "Starved bees", n = 76; Nic-Nic vs Nic-Suc $\chi^2 = 0.4$, $P = 0.5$; Nic-Nic vs Suc-Suc $\chi^2 = 0.1$, $P = 0.7$; Nic-Suc vs Suc-Suc $\chi^2 = 0.01$, $P = 0.9$) (Dataset 3). Furthermore no effect of colony of origin on mortality was found (GLM, "*ad libitum* food bees": F = 1.4, df = 2, $P = 0.24$; "Starved bees": GLM, F = 2.02, df = 2, $P = 0.14$). The median lethal time LT50 for the three groups did not differ ("*ad libitum* food bees": Suc-Suc LT50: 22 days, Nic-Suc LT50 = 23, days, Nic-Nic LT50 = 22; "Starved bees": Suc-Suc LT50: 25 hours, Nic-Suc LT50 = 28 hours, Nic-Nic LT50 = 31 hours).

GLMM analysis revealed significant main effects of treatment (df = 2, F = 3.46, $P = 0.03$) and time since inoculation (df = 1, F = 57.3, $P < 0.001$) on infection intensity, but not colony of origin (df = 2, F = 1.64, $P = 1.96$). No interaction between diet, time and colony was significant. Overall bees caged in Petri dishes consumed less food over the entire duration of the experiment if exposed to nicotine (Anova test: F = 9.68, n = 90, df = 2, 87, $P = 0.001$; Dunnett T3 post hoc test: Suc-Suc vs Nic-Nic and Suc-Suc vs Nic-Suc $P < 0.001$) (Dataset 4). Infected bees showed a slight preference (54 ± 17 %) for sucrose solution laced with nicotine (Paired samples t test, t = 2.14, df = 29, n = 30, $P = 0.04$).

Overall these findings indicate that, even though nicotine reduces the parasite load in infected bees, and such bees have a slight preference for sucrose solution laced with the alkaloid, there is no net benefit in term of survival for infected bees.

Dataset 3. Trade-off between detrimental and beneficial effects of nicotine

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Dietary nicotine effect on parasite load and life expectancy in infected bumblebees.

Dataset 4. Diet preference of caged bees

http://dx.doi.org/10.5256/f1000research.6262.d44614

Caged infected bee preference for nicotine-laced nectars.

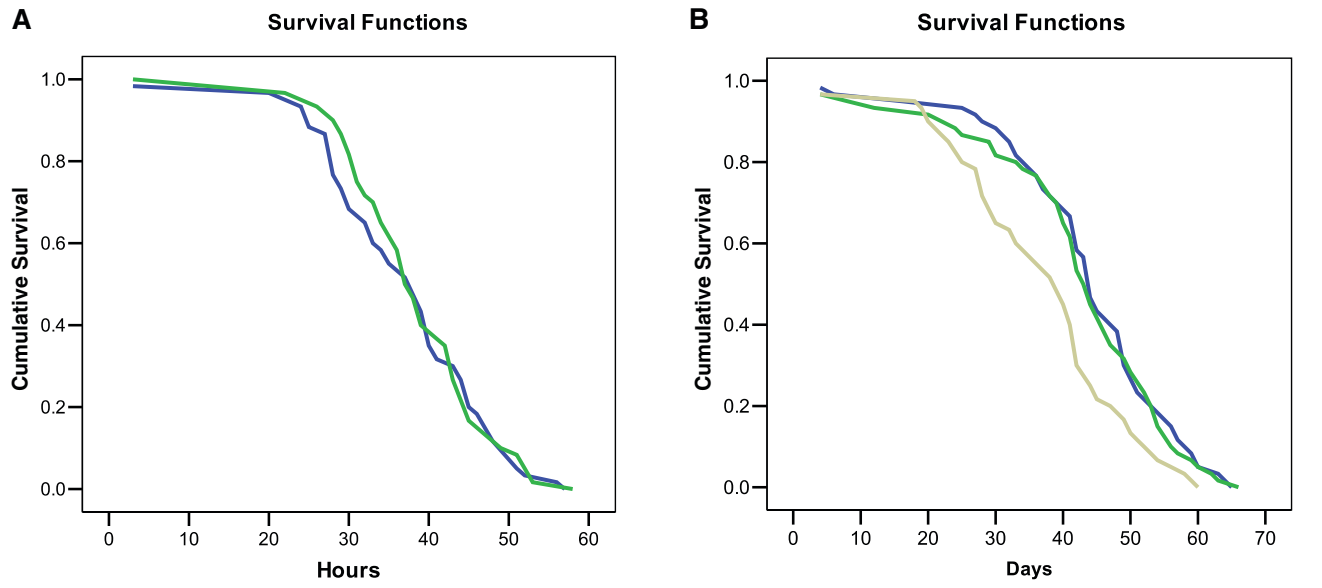

Figure 3. A: Cumulative survival of bees fed with a sucrose solution with (blue line) or without (green line) nicotine and starved. **B**: Cumulative survival of bees that received a daily diet of sucrose solution with (beige line), or without nicotine (blue line), or a single dose of nicotine on day one (green line).

Preference of freely flying bees for nicotine-laced flowers

Infected bumblebees allowed to forage on plastic flowers showed a significantly increased propensity to visit nicotine rewarding flowers when compared to healthy bees (t test, n = 31, 28, t = -2.4, df = 57, P = 0.016; Figure 4) (Dataset 5). Indeed on 100 consecutive choices infected bees visited the nicotine flowers on average 64.5 ± 13.8 (s.d.) times while healthy bees visited them 54.8 ± 19.4 (s.d.) times. Since test bees were introducing nicotine into the colony throughout testing, we controlled for prior exposure to nicotine effect on nicotine preference. Bees tested later in the experiment did not show a higher or lower nicotine preference (Spearman test, Infected bees: ρ = -0.21, n = 31, P = 0.3; Control bees n = 28, ρ = 0.041, P = 0.8). There was no correlation between pathogen load and the propensity of infected bees to visit flowers with nicotine-rich artificial nectar (Spearman test: n = 31, ρ = 0.19, df = 29, P = 0.28).

Figure 4. Percentage of preferred flowers rewording with nicotine-rich artificial nectar by infected bees (n = 31) and healthy bees (n = 28), (t test, P = 0.016). Infected bees visited nicotine-containing flowers 64.5 ± 13.8 (s.d.) times while healthy bees visited them 54.8 ± 19.4 (s.d.) times.

Dataset 5. Behavioural test

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Percentage of preferred flowers rewording with nicotine-rich artificial nectar by infected and healthy bees.

Discussion

Here we demonstrate that parasitized bumblebees modify their diet preference and foraging behaviour to delay the development of an infection. In our experimental setup the parasite infection induced an increased consumption of nicotine both in individually caged as well as in foraging bumblebees. Despite this preferential ingestion of a "non-nutritive" antimicrobial alkaloid by infected bees, the self-medication behaviour is not efficient since dietary nicotine does not fully cure *C. bombi* infection. Nonetheless bumblebees exhibited a reduced *C. bombi* load after daily consumption of the alkaloid. In nature, infection entails an array of costs (Alghamdi *et al.*, 2008; Brown *et al.*, 2000; Brown *et al.*, 2003; Gegear *et al.*, 2006). As a consequence, any reduction in the severity or progression of infection in bees, induced by mechanisms such as the consumption of nectar containing curative alkaloids (e.g. gelsemine (Manson *et al.*, 2010), anabasine and nicotine (Richardson *et al.*, 2015)), might be beneficial in terms of fitness for both bees and colonies.

In the same way as bumblebees have adapted their foraging behaviour to reduce the uptake of parasites (Fouks & Lattorff, 2011), bumblebees may be adapted to modify their diet with curative nectars once infected. The recent demonstration that honeybee nurse bees, infected with the microsporidian gut parasite *Nosema ceranae*, show different preferences for various types of honeys in a simultaneous choice test, preferring honeys with a higher antibiotic activity (Gherman *et al.*, 2014), suggests that such behaviours may be widespread in social pollinators. However, our results suggest that a description of this behaviour as pharmacophagy may require further evidence. Indeed, although dietary nicotine slows the progression of infection by a few days, this effect does not induce any benefit in terms of life expectancy of infected bees. Even if we cannot completely exclude that the weak effect of nicotine is due to the initial challenge being too strong for the nicotine to have a measurable influence on life expectancy, both nicotine concentration and *Crithidia* inocula used in our study simulated natural doses. Additional field and mesocosm tests are thus needed to clarify the actual benefits of ingestion.

Nicotine also has a costly effect on uninfected individuals, as shown by our toxicological assays. A daily diet containing nicotine, lasting more than two months, reduced the life expectancy of bumblebees, and this effect was stronger in smaller bees. This might possibly be aggravated in the wild, where bees are exposed to other stressors and do not have access to *ad libitum* food. However, we note that differences in mortality rate between controls and nicotine-treated bees started to be evident only after 20 days from the first exposure suggesting that in nature this detrimental effect may be mitigated due to the relatively short lifespan of foragers in the wild (da Silva-Matos & Garófalo, 2000). Moreover, in nature, bees may not forage on a single nectar source continuously for weeks as we simulated in our experiments, further reducing the negative effect of nicotine intake. In infected bumblebees the detrimental effect of nicotine is no longer evident suggesting that detoxification costs might be counterbalanced by the advantages in slowing the progression of the infection. However, contrary to our prediction, we found no trade-off between costs and benefits in terms of survival, and infected bumblebee lifespan was not affected by the consumption on nicotine. Similar results have recently been found for the antimicrobial alkaloid anabasine that did not induce a significant fitness benefit in the bumblebee species *B. impatiens* despite its effectiveness in reducing the parasite load by up to 80 percent (Richardson *et al.*, 2015).

The cost imposed by the consumption of nicotine in our experiments may explain why healthy bees did not constantly consume high doses of nicotine (Tiedeken *et al.*, 2014). Similarly, infected bees kept in Petri dishes reduced the overall uptake of food if exposed to nicotine. This is surprising given that those bumblebees also had a

slight preference for sucrose solution laced with the alkaloid, and free-flying healthy bumblebees were not repelled by artificial nectar laced with nicotine. While these behavioural preferences may be explained by the impact that some nectar alkaloids, including nicotine, have on learning and memory in bees (Chittka & Peng, 2013; Thany & Gauthier, 2005; Wright *et al.*, 2013), the mechanism behind the overall reduced consumption caused by nicotine remains unexplained. In humans at least, it is well established that nicotine has appetite-reducing effects (Jessen *et al.,* 2005).

Currently it is unclear how nicotine acts on *C. bombi*. Nicotine is a highly toxic molecule (Benowitz, 1998) that acts against a wide spectrum of bacterial and fungal pathogens (Pavia *et al.*, 2000). House sparrows and several finch species, for example, add smoked cigarette butts retaining substantial amounts of nicotine to their nests to reduce mite infestations (Suárez-Rodríguez *et al.*, 2013). While our *in vivo* microbiological experiments prove the antimicrobial activity of nicotine against the pathogen when ingested, they also suggest that nicotine does not directly interfere with the protozoan's viability, at least when measured as infectivity. As suggested by Manson *et al.* (2010), who found similar effects of the natural alkaloid gelsemine, an alkaloid-rich diet might increase a bee's excretion rate, as occurs for nectarivorous bird (Tadmor-Melamed *et al.*, 2004), effectively "flushing" *C. bombi* cells from the gut. Another possibility might be that nicotine, or perhaps its metabolites, directly modify the mid-gut epithelium or the environment of its lumen, making it less suitable for the parasite.

In conclusion, we believe that our results suggest that a more careful approach to interpreting impacts of plant metabolites on insect parasites is warranted. Recent findings have suggested that the preferential ingestion of natural nectar secondary metabolites in pollinators might play a key role in mediating pathogen transmission within and between colonies (Richardson *et al.*, 2015) or interactions among pollinators and their parasites (Manson *et al.*, 2010). Similarly, our results and other recent studies (Gherman *et al.*, 2014; Richardson *et al.*, 2015) have suggested that bees may self-medicate by consuming plant secondary metabolites when they are infected with parasites. However, our study suggests that the conditions under which nicotine consumption provides benefits to either bees or plants remain to be identified. The contention that secondary metabolites in nectar may be under selection from pollinators, or used by plants to enhance their own reproductive success (Chittka & Peng, 2013; Thomson *et al.*, 2014; Wright *et al.*, 2013), should ideally be confirmed with further studies, which examine the impacts of these metabolites on both bee and plant fitness under field-realistic conditions.

Data availability

F1000Research: Dataset 1. Infection experiments, 10.5256/f1000research.6262.d44610 (Baracchi *et al.*, 2015a).

F1000Research: Dataset 2. Laboratory toxicity bioassays, 10.5256/f1000research.6262.d44612 (Baracchi *et al.*, 2015b).

F1000Research: Dataset 3. Trade-off between detrimental and beneficial effects of nicotine, 10.5256/f1000research.6262.d44613 (Baracchi *et al.*, 2015c).

F1000Research: Dataset 4. Diet preference of caged bees, 10.5256/f1000research.6262.d44614 (Baracchi *et al.*, 2015d).

F1000Research: Dataset 5. Behavioural test, 10.5256/f1000research.6262.d44615 (Baracchi *et al.*, 2015e).

Author contributions

D.B. conceived the study and carried out the experiments. D.B. and M.J.F.B. designed the experiments. LC made significant contributions to the interpretation of the data. All authors equally contributed in writing and revising the draft of the manuscript and have agreed the final content.

Competing interests

No competing interests were disclosed.

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Data Source

Baracchi D, Brown MJF, Chittka L: **Dataset 2 in: Weak and contradictory effects of self-medication with nectar nicotine by parasitized bumblebees.** *F1000Research.* 2015b.
Data Source

Baracchi D, Brown MJF, Chittka L: **Dataset 3 in: Weak and contradictory effects of self-medication with nectar nicotine by parasitized bumblebees.** *F1000Research.* 2015c.
Data Source

Baracchi D, Brown MJF, Chittka L: **Dataset 4 in: Weak and contradictory effects of self-medication with nectar nicotine by parasitized bumblebees.** *F1000Research.* 2015d.
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Referee Report 20 April 2015

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Determining the extent that bumble bees may self-medicate by consuming floral nectar containing antimicrobial secondary plant metabolites when infected with a pathogen is a fascinating line of study. The experiments presented are well conducted and analyzed, and I found the results not entirely surprising or contradictory.

There was a temporary effect of nicotine (when provided in sugar syrup) against *Crithidia bombi* in *Bombus terrestris* workers. This effect might have been stronger and more long lasting, and even might have cleared the infection, if the dose of nicotine consumed was higher. This could be tested in future experiments using the high- and low-end concentrations found in floral nectar. Even though the infected bees preferred sucrose laced with nicotine in the clever foraging tests and in petri dishes, the authors say the results should be interpreted with caution because the life expectancy of the infected, nicotine-fed bees was not increased relative to controls. But as they also point out, bees don't live very long, and many factors affect worker bee life expectancy. Possibly, at the colony level, reducing the infection level even for a few days in a certain number of workers might slow the rate of horizontal transmission among nest mates; this remains to be tested.

I think it would be revealing to test the effects of these alkaloids, or other plant metabolites, on infected queen bumble bees. As *Crithidia* is also vertically transmitted, it would be interesting to know if the vertical transmission of this parasite could be reduced if the queen ingests nectar that contains antimicrobial metabolites. It also would be interesting to know if the infected workers that collect nectar containing these compounds feed the queen with them, potentially lowering her pathogen load, which would then might allow her to produce more reproductive male and gynes. These types of experiments are more difficult, but could yield more field-relevant results.

Yet, the experiments presented here are great first steps in understanding a new area of research on the interaction of pollinators, pathogens and plant compounds. I greatly appreciate that the authors offer a cautionary stance in interpreting their results, but I do think, given the physiological tradeoffs involved in consuming potentially toxic compounds, that small temporary effects on individual bees might translate to larger effects at the colony-level.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 10 April 2015

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The authors presented a fairly comprehensive set of experiments in order to elucidate the role that nicotine may have in self-medication of bumblebees against *Chrithidia* infection. The experiments progress well, starting with controlled lab infection studies to lab-based diet and lifespan analysis to a semi-field foraging test. I think the authors sufficiently described the methods and subsequent results and mostly had an appropriate discussion of the relevant findings. I just have a few small issues that the authors could address to improve and clarify aspects of the manuscript. But overall it is a strong paper.

First, I do think “contradictory” could be removed from the title as I don’t think the results are necessarily contradictory, but do just show a weak effect. Just because the ingestion of nicotine reduces parasite infection and does not have a subsequent effect of increasing lifespan of infected bees, this does not mean that there are no other fitness-related benefits or that at different doses effects may not be more pronounced. I think “weak effect” aptly describes the findings.

Generally, tables and figures could be labeled more effectively. For example, I think the fact that Figure 1 and 2 have the same x-axis is problematic given what the experimental treatments were. I think in Figure 2 it would be better to indicate that the Nicotine there is the Nicotine pre-treatment. Same for Table 2. Whereas in Figure 1 it would be nicotine diet. The legend for Figure 3 should explicitly state that these bees were all uninfected, since this is an important point, and since the lifespan data of infected bees is not represented in a figure.

For the discussion and results, overall I think there just are a few other points that can be made. One is simply drawing stronger connections across the multitude of tests that were done.

Infection experiments: would it be valuable to show statistically that there is no difference between the pre-treated *Chrithidia* and the standard (basically comparing figure 1 and figure 2)? Also is the 2-hour exposure time relevant? How was this decided upon? Same for dose of exposure. I think this needs to be discussed more thoroughly or at least a citation provided to justify this amount.

Trade-off: Important to note that *Chrithidia* infection was found to reduce lifespan of bees (as compared to the bees in the toxicity part of the study).

I think a discussion of the relevance of the doses used is important. Were any dose-response trials conducted? Presumably at a higher dose, it would be even more toxic to the bees and lower doses may not have much of an effect.

Could ingestion of nectar alkaloids be a generalized response to sickness? So maybe it’s not as effective against *Chrithidia*, but this isn’t the only parasite bumblebees get.

A larger context into how this might influence colony dynamics and health is important. This is hinted at in a couple sentences, but since all of these studies were really done with individual bees or individual

behavior, this is a significant point. Perhaps lifespan analysis of forager bumblebees would be different in a social setting. Similarly perhaps fitness benefits aren't seen since it's just measured in terms of individual lifespan, but maybe a reduction in parasite load affects foraging efficiency or nursing ability and thus colony productivity.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 27 March 2015

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Content of paper. This paper reports a set of nicely designed experiments aimed at determining whether *Bombus terrestris* self-medicates against trypanosome (*Crithidia bombi)* infection by seeking and consuming nicotine-laced floral nectar. Using captive bee colonies and artificial flowers in laboratory conditions permits well-controlled manipulative experiments that would be effectively impossible in the field. The results are ambivalent, in that dietary nicotine does reduce the intensity of gut infection, and infected bees do seek out nicotine, but attempts to demonstrate improved health in terms of worker bee lifespan yield negative results. For the most part, I endorse both the interpretation of these results and the recognition by Baracchi *et al.* that further research is needed to settle the question of self-medication.

I do have three reservations about the interpretations offered here, however, and I think the paper would be improved by some additional discussion of these issues. First, any anti-parasitic medicinal effects of toxic compounds will depend on dose rates. Too much medicine may harm the host; too little may exert no therapeutic effect. Baracchi *et al.* state that they have used "natural" dosages of nicotine, but what that means is that they have prepared solutions whose nicotine levels match those reported from some floral nectars. That is an appropriate starting point, but there may be little correspondence between the concentrations that flowers offer and those that bees are exposed to in a natural colony. Foraging workers typically collect floral nectar from various plant species that differ in nectar chemistry. Much of what they collect is not digested by the collectors themselves, but is transported back to the nest and regurgitated into communal honeypots that serve as energy stores for the larvae, the queen, and the many workers that do not forage. As this complicated cocktail is assembled, soluble compounds are concentrated by evaporation, diluted by mixing with other nectars, and probably further modified by enzymatic and microbial action. There is no reason to expect that the concentration of any particular compound in this brew bears any relationship to its concentration in one of the many floral nectars that have been pooled. Indeed, honeypots within a nest may hold different mixtures because particular foragers tend to discharge their collections into particular honeypots. I believe that explicit attention must be paid to honeypot composition if the question of bee medication is to advance.

Second, by choosing to look at toxic effects on workers, Baracchi *et al.* are not able to detect possible benefits of medication on other members of the colony, specifically larvae or the queen. One can imagine that certain inputs of nicotine to the colony might have no net effects of worker survival but might allow the queen to lay more eggs or the larvae to prosper. Indeed, effects could conceivably be harmful for the foragers but still beneficial to the colony. There might also be different effects on workers that forage and

those that serve as nurses. The social nature of these bees must be considered.

Third, by considering nectar only, Baracchi *et al.* don't consider the probability that secondary metabolites found in nectar are also likely to occur in the pollen of the same flower species. In nature, therefore, bees that choose to forage on nicotine-rich nectars are also likely to be collecting nicotine rich pollen from those flowers. To the extent that such correlations hold in nature, the larvae (who are the primary consumers of that pollen) may be receiving very different doses than adult bees.

In summary, it would be very illuminating, although tedious, to consider experiments that measure whole-colony health as a response variable rather than worker longevity.

Errors. In this review process, the lack of line numbers, or an editable form of the MS, makes it hard to flag such things as unclear phrases. Here are a few mistakes that should be addressed, however:
In the abstract, the plant family *Solanaceae* is misspelled.
In the caption for Figure 1, "bees received" should be rephrased.
I believe that there must be a serious error in Dataset 2. Unless I am missing something, the data reported for "starved" and "*ad libitum*" treatments are identical. This looks like a cut-and-paste error.

Column headings in the tables and data sets should be more explanatory. For example, in Table 2, experimental treatments are denoted simply as "time" and "nicotine." Those labels are too cryptic.
The "Statistical Analysis" section refers to several "classical...parameters," but these are not picked up in the Results. Reconcile?

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: Statement regarding conflict. I have two connections to this research. First, Lars Chittka did a stint of postdoctoral work in my lab at Stony Brook; we collaborated on several papers in the 1990s, and co-edited a book in 2001. We correspond occasionally but have not had an active collaboration since then. Second, I later supervised postdoctoral research on bumble bee disease and nectar chemistry by Robert Gegear, dissertations by Michael Otterstatter and Jessamyn Manson, and undergraduate research by Miruna Draguleasa. I share authorship on several of their papers that are cited prominently by Baracchi et al.