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CORRECTION

Correction: Bumble bees regulate their intake of essential protein and lipid pollen macronutrients

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There was an error published in *J. Exp. Biol.* **219**, 3962-3970.

The *x*-axis and *y*-axis of Fig. 1 were incorrectly labelled. The corrected figure is given below.

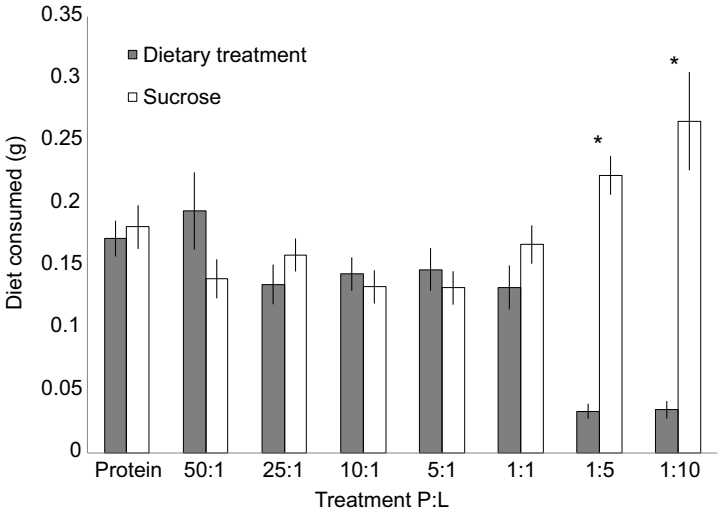

Fig. 1. Daily consumption of diets across treatments for *B. terrestris* foragers in single P:L diet assay. Treatments are represented by their protein:lipid (P:L) treatment diet ratio, including protein-only diets. Diets are represented as sucrose-only and diet associated with each treatment. Asterisks represent significant differences ($P<0.05$) in diet consumed within treatment. $N=15$ bees per treatment, data presented as means±s.e.m.

We apologise to the authors and readers for any inconvenience this may have caused.

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RESEARCH ARTICLE

Bumble bees regulate their intake of essential protein and lipid pollen macronutrients

A. D. Vaudo[1,*], D. Stabler[2], H. M. Patch[1], J. F. Tooker[1], C. M. Grozinger[1] and G. A. Wright[2]

ABSTRACT

Bee population declines are linked to the reduction of nutritional resources due to land-use intensification, yet we know little about the specific nutritional needs of many bee species. Pollen provides bees with their primary source of protein and lipids, but nutritional quality varies widely among host-plant species. Therefore, bees might have adapted to assess resource quality and adjust their foraging behavior to balance nutrition from multiple food sources. We tested the ability of two bumble bee species, *Bombus terrestris* and *Bombus impatiens*, to regulate protein and lipid intake. We restricted *B. terrestris* adults to single synthetic diets varying in protein:lipid ratios (P:L). The bees over-ate protein on low-fat diets and over-ate lipid on high-fat diets to reach their targets of lipid and protein, respectively. The bees survived best on a 10:1 P:L diet; the risk of dying increased as a function of dietary lipid when bees ate diets with lipid contents greater than 5:1 P:L. Hypothesizing that the P:L intake target of adult worker bumble bees was between 25:1 and 5:1, we presented workers from both species with unbalanced but complementary paired diets to determine whether they self-select their diet to reach a specific intake target. Bees consumed similar amounts of proteins and lipids in each treatment and averaged a 14:1 P:L for *B. terrestris* and 12:1 P:L for *B. impatiens*. These results demonstrate that adult worker bumble bees likely select foods that provide them with a specific ratio of P:L. These P:L intake targets could affect pollen foraging in the field and help explain patterns of host-plant species choice by bumble bees.

KEY WORDS: Foraging behavior, Geometric framework, Nutrient regulation, Nutritional ecology, Pollination, Pollinator health

INTRODUCTION

Bee population declines are linked to many interacting factors associated with anthropogenic land-use intensification (Goulson et al., 2015; Ollerton et al., 2014), including the reduction of host-plant abundance and diversity, which might lead to nutritional stress for some bee species (Biesmeijer et al., 2006; Carvell et al., 2006; Potts et al., 2010). Differences in resource quality can have direct effects on bee development, reproduction, immunocompetence, resilience to stress, and survival (Vaudo et al., 2015). Therefore, to address the problem of nutritional deprivation in the landscape, it is crucial to develop a comprehensive understanding of the nutritional requirements of bees.

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Bees obtain their macronutrients (carbohydrates, proteins and lipids) from floral nectar and pollen; pollen provides proteins and lipids, whereas bees primarily obtain carbohydrates from nectar (Nicolson and Thornburg, 2007) to fuel energetically costly foraging efforts, and adults cannot survive without a continuous carbohydrate source (Brodschneider and Crailsheim, 2010). Differences in protein content in bee diets can influence adult reproduction, physiology, immunity and larval development (Alaux et al., 2010; Cardoza et al., 2012; Di Pasquale et al., 2013; Génissel et al., 2002; Human et al., 2007; Li et al., 2012; Tasei and Aupinel, 2008a). For bees, lipids play important roles in production of cuticular hydrocarbons and wax, behavioral maturation in adults (through the reduction in lipid stores), diapause, learning, and development of glands that produce brood food (Canavoso et al., 2001; Fliszkiewicz and Wilkaniec, 2007; Toth et al., 2005). Essential sterols obtained exclusively from pollen are precursors for molting hormone, which is essential for larval development (Feldlaufer et al., 1986; Roulston and Cane, 2000; Vanderplanck et al., 2014). Moreover, the lipid-enriched pollenkitt on the exterior of pollen is an important discriminative stimulus and phagostimulus of pollen for bees (Dobson and Bergström, 2000; Pacini and Hesse, 2005).

Although bees can obtain protein and lipids from most pollen sources, pollen protein (including essential amino acids) and lipid (including essential fatty acids and sterols) concentrations vary considerably among plant species, with pollen typically containing ∼2–60% protein and ∼2–20% lipid (Roulston and Cane, 2000). Inequality of nutrients among plant species implies that bees might selectively forage for pollen to meet their nutritional demands. Generalist bumble bee species, such as *Bombus terrestris* (Linnaeus 1758) (Hymenoptera: Apidae) in Europe, North Africa and the Middle East, and *Bombus impatiens* Cresson 1863 in North America, forage on a variety of different plant species during their lives. A handful of studies have suggested that bumble bees preferentially forage on flowers that have high sugar concentrations in nectar (Cnaani et al., 2006; Somme et al., 2015), and high protein (Cardoza et al., 2012; Hanley et al., 2008; Konzmann and Lunau, 2014) or amino acid and sterol content in pollen (Somme et al., 2015). A recent study demonstrated that *B. impatiens* – both when foraging for colonies with brood or isolated from brood – preferentially forage for pollen with high protein:lipid ratios and consume different amounts of pollen diets depending on protein and lipid concentrations (Vaudo et al., 2016). This indicates that bees are sensitive to both protein and lipids in their diet and are likely to exhibit nutrient regulation that affects their feeding behavior.

Although foraging bumble bees collect pollen mainly to feed developing larvae, adult workers also eat pollen (Brodschneider and Crailsheim, 2010; Roulston and Cane, 2000) when they assess nutritional stores in pollen pots (Dornhaus and Chittka, 2005), while they feed pollen to larvae (Pereboom, 2000; Pereboom et al., 2003) or when they eat pollen to develop their own ovaries for male

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 3962-3970 doi:10.1242/jeb.140772

egg laying (Amsalem et al., 2015; Tasei and Aupinel, 2008a). In three-worker queenless microcolonies, workers were shown to eat between 0.4 and 0.9 g of pollen in the 5 days prior to egg laying, which would average ∼25–60 mg day^{-1} of pollen by worker egg-layers (Tasei and Aupinel, 2008a,b).

Many studies have demonstrated that insects regulate their consumption of food around optimal proportions of macronutrients in ways that reflect their age, somatic needs and reproductive status (Behmer, 2009; Simpson and Raubenheimer, 1993; Simpson et al., 2004). The geometric framework (GF) for nutrition is a method for examining the mechanisms and constraints that govern how animals regulate feeding to achieve specific macronutrient optima, or 'intake targets'. It employs an approach wherein individuals self-select diets or alter food consumption when confined to diets comprising specific ratios of macronutrients (Raubenheimer and Simpson, 1999; Simpson and Raubenheimer, 1993, 2012). The GF has been successfully used to characterize nutrient balancing for protein and carbohydrate in worker honey bees (Altaye et al., 2010; Paoli et al., 2014; Pirk et al., 2010) and bumble bees (Stabler et al., 2015). Workers, especially foragers, have a high demand for carbohydrates, as reflected in their measured intake targets, which for bumble bees is ∼1:150 protein:carbohydrate (P:C) ratio. Moreover, their tolerance of dietary protein (or essential amino acids) is relatively low, as they have reduced survival when forced to ingest surplus protein (Altaye et al., 2010; Paoli et al., 2014; Pirk et al., 2010; Stabler et al., 2015). This has also been observed in ants (Dussutour and Simpson, 2012) and fruit flies where there is a survival cost of ingesting protein to maximize reproduction (Lee et al., 2008).

None of the previous studies using the GF have tested whether bees or other social insects regulate their dietary intake of fats. The few studies that have investigated protein and fat regulation in insect herbivores have been limited to lepidopteran larvae, but were not clear assessments that used the GF to investigate simultaneous regulation of both nutrients (Stockhoff, 1993; Thompson and Redak, 2005). In contrast, arthropod predators clearly regulate both protein and fat simultaneously. For example, the ground beetle *Agonum dorsale* adjusts its consumption of complementary foods to meet an intake target of proteins and fat (Mayntz et al., 2005; Raubenheimer et al., 2007). Similarly, the wolf spider *Pardosa prativaga* was shown to regulate its diet by eating flies that complemented a previous diet higher in protein or fat (Mayntz et al., 2005), and over-ate protein on lipid-poor diets to reach an intake target for lipid (Jensen et al., 2011).

Here, we use the GF methodology to test and measure regulation of protein and lipid intake in bumble bee foragers of two species, *B. terrestris* and *B. impatiens*, both important crop pollinators and commercially available in their respective geographic range (Velthuis and van Doorn, 2006; Amsalem et al., 2015). In our first experiment, we restricted *B. terrestris* individuals to single synthetic diets differing in P:L ratios that spanned the realistic and extreme possibilities found in pollen, and measured their food consumption and survival. Next, using the results of the first experiments to select appropriate diets, we presented *B. terrestris* and *B. impatiens* individuals with two diets differing in their P:L ratios to determine if the two species indeed regulate protein and lipids to a specific intake target. We expected that the bees of each species would regulate their P:L intake to a target at which they survived best. We also expected that the bumble bees would defend a carbohydrate target, given the importance of carbohydrates for bees. Our results characterize the specific macronutrient requirements of these two species and provide insights into the ability of bumble bees to regulate lipids in their diet, suggesting nutritional quality might drive pollen foraging preferences.

MATERIALS AND METHODS

General bee-rearing conditions

We purchased mature research colonies of *Bombus terrestris* ('single P:L diet assay' and 'paired P:L diets assay') and *B. impatiens* (paired P:L diets assay) from Koppert Biological Systems (Havervill, Suffolk, UK for *B. terrestris*; Howell, MI, USA for *B. impatiens*). Each colony contained ∼100 workers and the natal queen. During the course of the study, we stored colonies at ambient temperatures and provided them sugar water *ad libitum*. For each assay, we collected foragers as they exited their colonies and placed individual bees in their own 11×11×10 cm plastic cages kept in a 24 h dark incubator at 28°C and 40% humidity. We provided all diets to bees in 2 ml microcentrifuge tubes with four holes drilled in the tube from which the bees could feed. The tubes were suspended halfway up and at opposite sides of each cage such that the bees could perch on the tube and feed through the holes. We first performed the single P:L diet assay with *B. terrestris* in the UK. Based on the results of this assay, we designed the paired P:L diets assay to be sensitive for both bumble bee species as we expected that their intake targets are not radically different. We conducted the paired P:L diets assay for *B. terrestris* in the UK, and *B. impatiens* in the USA.

Single P:L diet assay

Individual forager *B. terrestris* bees (15 bees per treatment, four colonies) were given access to food tubes containing 0.5 mol l^{-1} sucrose solution or 0.5 mol l^{-1} sucrose solution containing a specific protein:lipid ratio (P:L). We tested eight different dietary ratios of P:L (protein-only, 50:1, 25:1, 10:1, 5:1, 1:1, 1:5 and 1:10; Table 1). The sucrose-only food source was necessary to allow bees to reach their high carbohydrate demand and needed to be separate for bees to freely consume it without consuming proteins and lipids; omitting sucrose would cause high mortality (Brodschneider and Crailsheim, 2010). This also provided a simulation of what bees actually experience by providing a carbohydrate-only source or 'nectar' and a fixed protein/lipid/sugar source or 'pollen'. Protein was held constant while we adjusted the lipid concentration. We chose these particular P:L diets to include possible ranges of P:L ratios in pollen (Roulston and Cane, 2000) as well as values outside

Table 1. Diet composition

Nutrient source	Sucrose-only	Protein-only	100:1	75:1	50:1	25:1	10:1	5:1	1:1	1:5	1:10
Sucrose (g)	171	17.1	17.1	17.1	17.1	17.1	17.1	17.1	17.1	17.1	17.1
Casein (g)	–	0.342	0.342	0.342	0.342	0.342	0.342	0.342	0.342	0.342	0.342
Lecithin (g)	–	–	0.00342	0.00456	0.00685	0.0137	0.0342	0.0685	0.342	1.71	3.42
H_2O (ml)	1000	100	100	100	100	100	100	100	100	100	100

Diets are represented by their protein:lipid (P:L) ratios or sucrose-only and protein-only diets. Sucrose was used as the carbohydrate source, soy lecithin was used as the lipid source, and casein as a protein source.

Journal of Experimental Biology (2016) 219, 3962-3970 doi:10.1242/jeb.140772

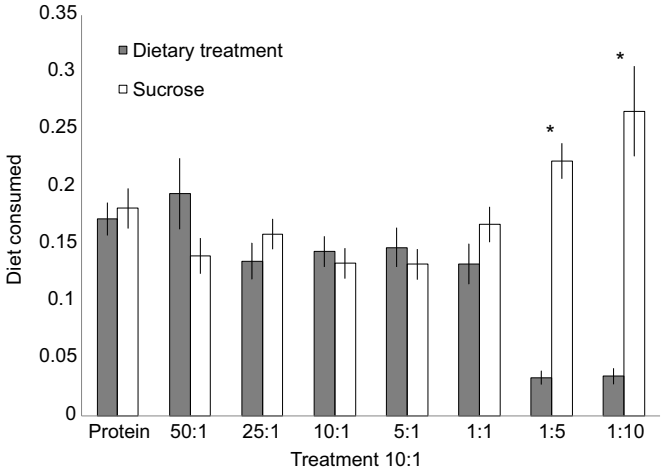

Fig. 1. Daily consumption of diets across treatments for *B. terrestris* foragers in single P:L diet assay. Treatments are represented by their protein:lipid (P:L) treatment diet ratio, including protein-only diets. Diets are represented as sucrose-only and diet associated with each treatment. Asterisks represent significant differences (P<0.05) in diet consumed within treatment. N=15 bees per treatment, data presented as means±s.e.m.

of the reported range of P:L in pollen. Nutrient sources were sucrose (Sigma-Aldrich, St. Louis, MO, USA) for carbohydrates, casein sodium salt from bovine milk (Sigma-Aldrich) for protein, and 100% soy lecithin (Optima Health & Nutrition, Bradford, UK) for lipids (>91% fat), which contains essential fatty acids (32% ω-6/linoleic acid, 4% ω-3/alpha-linolenic acid). Soy lecithin was chosen as the lipid source because it is an emulsifier and can be used for liquid diets. To prepare the diets, we mixed the lecithin into solution using a stir plate for ∼1–2 h under low heat. Liquid diets were used because they are easy for the bees to ingest and allow accurate measurement of consumption.

Experiments lasted 7 days, and we replaced each food tube daily. We weighed food tubes each day prior to placement in the cage and 24 h later. Cages with three tubes of each diet (replaced daily) and no bees served as controls to measure the daily evaporation rate for each diet. Amounts of solution (g) consumed by bees were adjusted by the daily mean amount of solution that had evaporated from the control cages prior to analysis. We calculated the mass of each nutrient (carbohydrate, protein or lipid) consumed from the total mass consumed from each diet tube each day. We measured the thorax width of each individual bee as a covariate in data analyses to control for the effect of size on diet consumption. We recorded the number of days each bee survived in the assay with a maximum of 7 days.

Table 2. Daily consumption of nutrients for *B. terrestris* foragers in single P:L diet assay

Treatment	Carbohydrate (mg)	Protein (mg)	Lipid (mg)
1:10	50±7^{a,b}	0.12±0.02^{b}	1.20±0.23^{a}
1:5	44±3^{b}	0.11±0.02^{b}	0.57±0.10^{b}
1:1	50±4^{a,b}	0.44±0.06^{a}	0.44±0.06^{b,c}
5:1	47±3^{a,b}	0.50±0.06^{a}	0.11±0.012^{c,d}
10:1	47±3^{a,b}	0.49±0.05^{a}	0.05±0.005^{d}
25:1	50±3^{a,b}	0.47±0.05^{a}	0.02±0.002^{d}
50:1	57±5^{a,b}	0.66±0.11^{a}	0.01±0.002^{d}
Protein-only	60±4^{a}	0.60±0.05^{a}	–

Daily consumption values are means±s.e.m. Treatments are represented by their protein:lipid (P:L) diet ratio, including protein-only diet. Means marked with different letters within each column are statistically different (P<0.05 by Tukey-HSD pairwise comparisons).

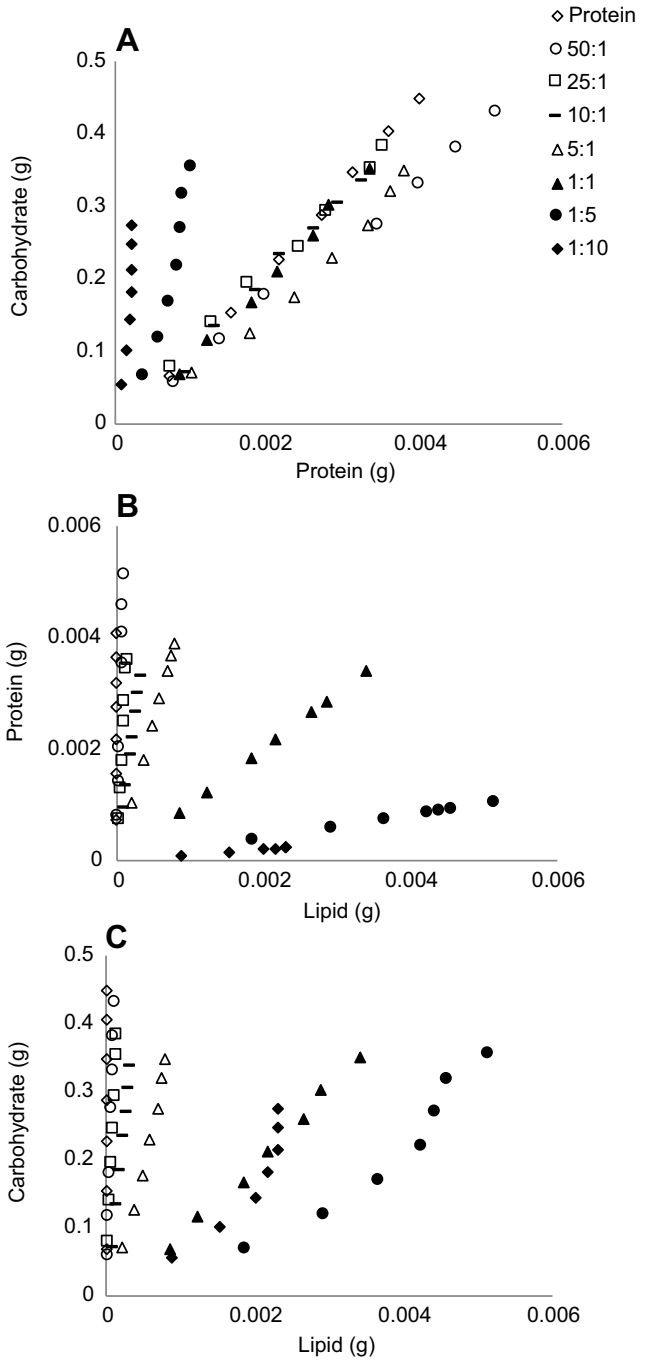

Fig. 2. Nutritional arrays of *B. terrestris* foragers surviving 7 days in single P:L diet assay. Treatments are represented by their protein:lipid (P:L) diet ratio, including protein-only diet. Markers of each treatment represent mean cumulative consumption of each nutrient for each successive day up to 7 days forming daily trajectories. (A) Carbohydrate and protein array, (B) protein and lipid array, and (C) carbohydrate and lipid array. $N_{Protein}$=10, $N_{50:1}$=9, $N_{25:1}$=9, $N_{10:1}$=11, $N_{5:1}$=10, $N_{1:1}$=8, $N_{1:5}$=7, $N_{1:10}$=4.

Paired P:L diets assay

To test our hypothesis that bumble bee intake targets lie within the 25:1–5:1 P:L range (see 'Single P:L diet assay' in Results), we measured survival and nutrient consumption of *B. impatiens* and

Journal of Experimental Biology (2016) 219, 3962-3970 doi:10.1242/jeb.140772

Table 3. Cox regression of survival for *B. terrestris* foragers in single P:L diet assay

Treatment	B	s.e.m.	χ^2	d.f.	*P*-value	Exp(B)	95.0% CI for Exp(B)	
							Lower	Upper
Protein			9.667	7	0.208			
50:1	0.266	0.606	0.193	1	0.661	1.305	0.398	4.275
25:1	0.186	0.606	0.094	1	0.759	1.204	0.367	3.946
10:1	–0.256	0.671	0.146	1	0.703	0.774	0.208	2.884
5:1	–0.019	0.632	0.001	1	0.976	0.981	0.284	3.389
1:1	0.375	0.586	0.410	1	0.522	1.455	0.462	4.584
1:5	0.372	0.570	0.425	1	0.514	1.451	0.474	4.436
1:10	1.136	0.540	4.424	1	0.035*	3.113	1.080	8.970

Treatments are represented by their protein:lipid (P:L) diet ratio, including protein-only diet. Protein-only diet (no lipid) was used as reference to test the effect of adding lipids to the diet. Note that likelihood of mortality (B) decreased for 10:1 treatment, and increased as the lipid content of the diet increased. Model: χ^2=10.52, d.f.=7, *P*=0.161. **P*>0.05.

B. terrestris foragers presented with paired P:L diets encompassing this range. As in the single P:L diet assay, we collected *B. impatiens* and *B. terrestris* foragers as they exited their colonies and caged them individually (20 bees per treatment; two colonies for each species).

For each treatment, we provided a bee with one of four paired P:L diets and with a sucrose-only food tube. These diet pairings were: (1) 25:1 and 5:1, (2) 50:1 and 5:1, (3) 75:1 and 5:1, and (4) 100:1 and 5:1 P:L (diets prepared as above; Table 1). We measured daily consumption of each diet and nutrient (accounting for evaporation rate) and survival of bees over 7 days (see 'Single P:L diet assay', above). Prior to placement in cages, we cold-anesthetized and weighed foragers to use their mass as a covariate in data analyses to control for effects of size on diet consumption. Note that thorax width and bee mass are correlated (Stabler et al., 2015) and we measured thorax width in the single P:L diet assay.

Statistical analysis

Single P:L diet assay

We conducted survival analyses with Cox-regression proportional hazards, and used the protein-only treatment as reference or control to determine the effect on bee survival of adding lipid to the diet. To determine whether bumble bees ate randomly among diet sources or if particular treatment diets caused differential feeding behavior, we analyzed differences in daily consumption of diet sources among treatments by two-way ANOVA and *post hoc* Tukey-HSD pairwise comparisons with treatment, diet source (treatment diet or sucrose-only) and the interaction of treatment and diet source as independent variables, and thorax width as a covariate. To analyze differences in daily consumption of nutrients among treatments, we used MANCOVA with *post hoc* Tukey-HSD pairwise comparisons with nutrient (carbohydrate, protein or lipid) as the dependent variable and thorax width as a covariate. Finally, for bees that survived on the diets for all 7 days, we analyzed differences in cumulative consumption of carbohydrate, protein and lipid with MANCOVA and *post hoc* Tukey-HSD pairwise comparisons with nutrient (carbohydrate, protein or lipid) as the dependent variable and thorax width as a covariate. After reviewing the data, it was apparent that there were differences in amounts of nutrients consumed between bees that died and survived in the 1:10 P:L treatment. We compared their cumulative consumption of nutrients on day three, using MANOVA and *post hoc t*-tests for each nutrient.

Paired P:L diets assay

Bombus terrestris and *B. impatiens* were analyzed separately. We analyzed differences in survival among treatments with the Kaplan–Meier test (because there was no reference group as above for Cox regression). To determine daily differences in mass of diets consumed among treatments, we conducted two-way ANOVA and *post hoc* Tukey-HSD pairwise comparisons, using treatment, diet source (5:1, treatment diet and sucrose-only), and the interaction of treatment and diet source as independent variables with colony and bee mass as covariates. Note that bee mass was used as a measure of size for this assay whereas thorax width was used in the single P:L diet assay. These are correlated metrics of bee size used as covariates for consumption per bee (Stabler et al., 2015). Finally, for bees that survived all 7 days, we analyzed cumulative nutrient consumption among treatments by MANCOVA with *post hoc* Tukey-HSD pairwise comparisons with nutrient (carbohydrate, protein or lipid) as the dependent variable and colony and bee mass as covariates. If consumption of each nutrient among treatments was similar, we could conclude that the bumble bees were regulating their nutrients equally. We determined P:C and P:L ratios consumed by bees using the average cumulative consumption of each treatment. All statistical analyses were conducted with JMP Pro v.12 (SAS Institute); SPSS Statistics (IBM) was used for Cox regression.

RESULTS

Single P:L diet assay

For 7 days, we fed *B. terrestris* foragers with sucrose-only and one of the P:L diets. The total quantities of food the bees consumed each day did not differ significantly across treatments ($F_{7,1321}$=1.99, *P*=0.053); the only pairwise difference was that foragers in the

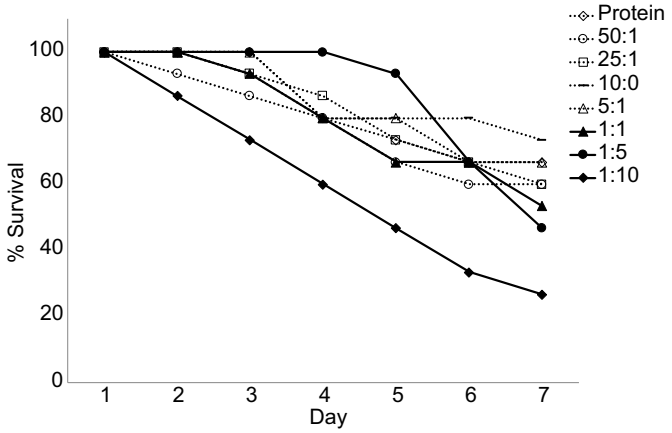

Fig. 3. Survival curve of *B. terrestris* foragers in single P:L diet assay. Treatments are represented by their protein:lipid (P:L) treatment diet ratio, including protein-only diet. Note that mortality increased as the lipid content of the diets increased. *N*=15 bees per treatment.

Journal of Experimental Biology (2016) 219, 3962-3970 doi:10.1242/jeb.140772

'protein-only' treatment ate more each day than bees on the high-fat 1:5 P:L treatment at $P<0.05$ (Fig. 1). Bees differed in the relative amounts of each diet (treatment diet versus sucrose-only) consumed (treatment×solution; $F_{7,1321}=16.0$, $P<0.001$; Fig. 1). Notably, bees consumed much less of the treatment diet than sucrose-only diet in the highest lipid treatments (1:5, 1:10 P:L; Fig. 1).

The only significant difference in daily consumption of carbohydrates was between protein-only and 1:5 treatments ($F_{8,666}=5.32$, $P<0.001$; Table 2), but bees across treatments differed significantly in amounts of protein and lipid consumed (MANCOVA: $F_{21,1640}=13.7$, $P<0.001$). Bees on the highest fat diets (1:5 and 1:10 P:L) consumed much less protein than the other treatments ($F_{8,663}=14.7$, $P<0.001$; Table 2), suggesting that they ceased eating the diet after having reached or exceeded their lipid intake target, and therefore did not reach their protein target. Finally, bees across treatments differed significantly in amounts of lipids consumed; specifically, bees consumed more lipids as lipid content of the treatment diet increased ($F_{7,573}=20.4$, $P<0.01$; Table 2).

For the bees that survived all 7 days of the experiment, there were significant differences among treatments in cumulative amount of nutrients consumed (MANCOVA: $F_{21,164}=5.03$, $P<0.001$; Fig. 2). Though there were no differences in cumulative carbohydrates consumed across treatments ($F_{7,59}=1.13$, $P=0.36$; Fig. 2A,C), bees on different diets consumed significantly different amounts of cumulative protein and lipids over 7 days. Similar to the daily consumption data, bees on the highest lipid treatments (1:5 and 1:10 P:L) consumed significantly less protein ($F_{7,59}=3.86$, $P=0.002$; Fig. 2A,B).

For cumulative lipids consumed, surviving bees in the 1:10, 1:5 and 1:1 treatments consumed significantly more lipids than bees on the remaining treatments ($F_{7,59}=10.2$, $P<0.001$; Fig. 2B,C). Furthermore, bumble bee foragers consumed on average ~3.5 mg protein on 1:1, 5:1, 10:1 and 25:1 P:L diets, while consuming ~5.1 mg protein on the 50:1 P:L diet ($F_{1,59}=2.86$, $P<0.1$), suggesting that bees compensated for low lipids by overeating the 50:1 diet to reach an intake target for lipid (Fig. 2B). These data also indicate that *B. terrestris* foragers regulated their protein intake, eating similar amounts of proteins (~4.0 mg) except on the highest lipid diets of 1:5 and 1:10 (~0.6 mg).

Bombus terrestris foragers had a greater risk of mortality when they consumed diets high in lipid (Table 3). Specifically, the mortality risk was lowest for the bees fed the 10:1 and 5:1 diets, whereas bees fed diets with proportionally greater quantities of lipids had increased risk of dying over 7 days (Table 3). Although bees in the high-fat treatment (1:5 P:L) seemed to survive well in the first days of the study, their mortality increased sharply over the remainder of the week and ended with the second-highest mortality and a nearly equal hazard ratio (Figs 1 and 3). Interestingly, by day three on the 1:10 P:L diet, surviving bees had eaten significantly less of their treatment diet (protein and lipid) than those bees that died ($t_{14}=2.29$, $P<0.02$), but living and dead bees ate equal amounts of carbohydrates ($t_{14}=0.64$, $P=0.27$; Fig. 4). These data suggest that high lipid consumption leads to toxicity and increased mortality.

Bombus terrestris foragers (1) over-ate lipids to defend their protein intake, (2) had increased mortality as lipid content of diets increased or decreased away from 10:1 P:L, and (3) increased protein consumption on the 50:1 P:L diet to potentially defend a lipid target. Therefore, we hypothesized that the P:L intake target of bumble bees lies within the 25:1–5:1 range. We performed a paired P:L diets assay to identify the actual intake target for P:L of *B. terrestris* and *B. impatiens*.

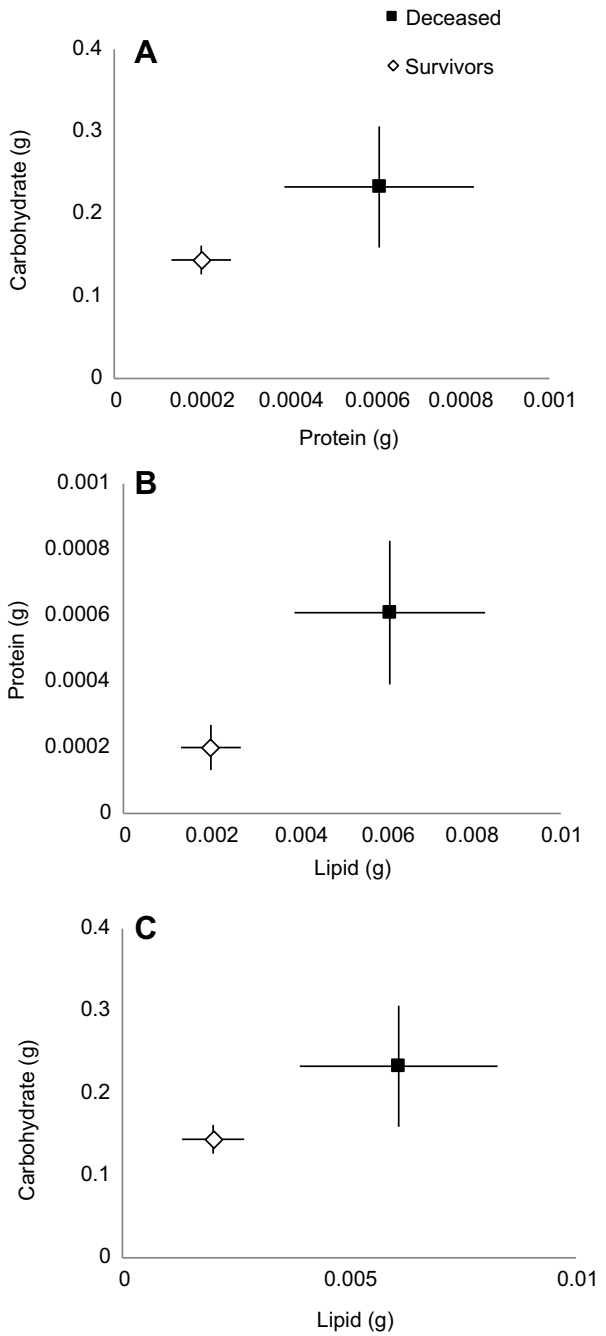

Fig. 4. Cumulative nutrient consumption of *B. terrestris* foragers on day 3 in 1:10 P:L treatment in single P:L diet assay. Mean±s.e.m. cumulative consumption of nutrients by deceased ($N=11$) and surviving ($N=4$) *B. terrestris* foragers. (A) Carbohydrate and protein, (B) protein and lipid, and (C) carbohydrate and lipid. Note that surviving bees ate significantly less protein and lipid than the deceased bees.

Paired P:L diets assay

For 7 days, we fed *B. impatiens* and *B. terrestris* workers a single sucrose-only diet, a 5:1 P:L diet, and a complementary treatment P:L diet (25:1, 50:1, 75:1 or 100:1). Each diet pairing of 5:1 P:L and treatment P:L created a protein and lipid nutrient space encompassing the hypothesized P:L intake target. The bees consumed significantly different amounts of total food across treatments (*B. impatiens*: $F_{3,1446}=5.65$, $P<0.001$; *B. terrestris*: $F_{3,1178}=4.75$, $P<0.003$), diet sources (*B. impatiens*: $F_{2,1446}=23.7$, $P<0.01$; *B. terrestris*:

Journal of Experimental Biology (2016) 219, 3962-3970 doi:10.1242/jeb.140772

$F_{2,1178}$=30.7, P<0.001), and the relative amounts of each diet source consumed among treatments (treatment×diet source interaction: *B. impatiens*: $F_{6,1446}$=3.55, P=0.0017; *B. terrestris*: $F_{6,1178}$=3.31, P=0.003; Fig. S1). Importantly, daily consumption differed between the treatment diet (25:1, 50:1, 75:1 100:1) and the 5:1 diet for both *B. impatiens* and *B. terrestris*, indicating that these diets were not being consumed randomly (Fig. S1).

Surviving *B. impatiens* and *B. terrestris* foragers, analyzed separately, regulated their carbohydrate, protein and lipid intake. Consumption of the three macronutrients and total nutrients across treatments was not significantly different within each species (carbohydrate: *B. impatiens*: $F_{3,52}$=2.20, P=0.10; *B. terrestris*: $F_{3,47}$=1.50, P=0.23; protein: *B. impatiens*: $F_{3,52}$=2.63, P=0.06; *B. terrestris*: $F_{3,47}$=1.02, P=0.39; lipid: *B. impatiens:* $F_{3,52}$=1.78, P=0.16; *B. terrestris*: $F_{3,47}$=0.02, P=0.99; total nutrients by MANCOVA: *B. impatiens*: $F_{9,122}$=1.35, P=0.22; *B. terrestris*: $F_{9,110}$=1.07, P=0.39; Table 4, Fig. 5; Fig. S2). Therefore, *B. impatiens* and *B. terrestris* foragers regulated their P:L intake to within our hypothesized range, averaging 12:1 P:L for *B. impatiens* and 14:1 P:L for *B. terrestris* (Table 4, Fig. 5; Fig. S2). The P:C intake targets regulated by both species averaged 1:85 P:C for *B. impatiens* and 1:67 P:C for *B. terrestris* (Table 4, Fig. 5; Fig. S2). Both bee species survived equally well on the various diets (*B. impatiens*: χ^2=3.98, d.f.=3, P=0.26; *B. terrestris*: χ^2=0.39, d.f.=3, P=0.94; Fig. S3).

DISCUSSION

Our experiments revealed that *B. terrestris* and *B. impatiens* regulated their protein and lipid intake to an average of 14:1 and 12:1, respectively, with *B. terrestris* preferring a diet slightly lower in fat than *B. impatiens*. Also, bees limited to diets high in lipids had increased risk of mortality (Table 3, Fig. 3). Taken together, this study provides the first evidence that pollinators (specifically *Bombus* spp. bees) regulate fat intake. Coupled with our previous study that demonstrated that bumble bee foraging preferences were significantly correlated with protein:lipid ratios in pollen (Vaudo et al., 2016), these results suggest that pollinators adjust their foraging to achieve specific macronutrient targets.

The protein and lipid regulation of bumble bee adults seems more similar to predaceous arthropods than herbivorous ones. *Manduca sexta* caterpillars, within a similar design to our paired P:L diets assay, failed to regulate lipid intake but preferred diets high in fat (Thompson and Redak, 2005). In contrast, both *B. terrestris* and *B. impatiens* workers regulated their intake of fat and preferred diets with specific P:L ratios. This difference is likely due to the vastly different life histories between lepidopteran larvae, which are typically constrained to specific food sources, and hymenopteran adults, which can forage among many sources. Two predaceous species (i.e. the wolf spider and ground beetle) ate protein excessively on low-fat diets, apparently to reach a lipid intake target (~4:1 P:L for wolf spider; or ~2:1 P:L for ground beetle; see Jensen et al., 2011; Mayntz et al., 2005; Raubenheimer et al., 2007). In our work, *B. terrestris* generally ate more protein on the low-fat diet (50:1 P:L) than the other treatments, including those that provided only protein. This behavior indicates that workers might also over-eat protein to reach their lipid intake; indeed, lipid intake did not differ across the groups fed 50:1, 25:1, 10:1 and 5:1 diets. Finally, the web-building spider *Stegodyphus lineatus*, having no control over the nutrient composition of prey captured in its web, selectively extracts dietary protein from prey based on previous feeding history (Mayntz et al., 2005). Bee larvae assimilate pollen protein and lipids efficiently (Roulston and Cane, 2000), but it remains to be tested if the sedentary and dependent bee larvae can differentially assimilate these nutrients to reach their intake targets or if they are completely dependent upon adults to sense and select an appropriate diet for them.

In contrast to the predatory ground beetle *A. dorsale*, which stopped eating when it reached its lipid intake target in high-fat diets (Raubenheimer et al., 2007), *B. terrestris* over-ate lipid in high-fat diets (1:1, 1:5 and 1:10 P:L), potentially to reach their protein target. This overconsumption of lipid to reach a protein target might have led to increased mortality. For example, bees survived when they ate less of the high-fat diet 1:10 P:L (Fig. 4). Additionally, although the bees in the 1:5 P:L treatment ate significantly less of the treatment diet than the sucrose-only diet, their high lipid consumption in the first days of the study likely lead to their rapid death (Figs 1–3). Thus, it seems that the surviving bees were able to eat enough to meet their nutritional needs, sense the toxicity of the diet, and cease feeding, whereas the others did not. What caused this individual variation in behavior remains to be determined; the bees used in this study were not age-controlled, and thus there might have been physiological differences associated with age, social status or behavioral task. Further, in attempt to regulate nutritional intake, the trend of over-ingesting diets at the cost of mortality has also been observed in *Spodoptera littoralis* caterpillars overeating carbohydrates on high-carbohydrate, low-protein diets (Raubenheimer et al., 2005).

Although feeding behavior might be affected by total nutrient concentration of the diets, we show that it was fat concentration or P:L ratios of the diets that influenced bee regulation of protein and lipid intake. In nearly all treatments in the single P:L diet assay the bees consumed similar quantities of total food. Thus, by fixing protein and adjusting lipid concentration in the diet, we demonstrated that the bees changed their feeding behavior to compensate for low fat in the diet, or suffered mortality attempting to reach a protein target. Combining this information with that of the

Table 4. Consumption by *B. impatiens* and *B. terrestris* foragers in the paired P:L diets assay and protein:carbohydrate (P:C) and protein:lipid (P:L) intake ratios over 7 days

	Treatment	Carbohydrate (mg)	Protein (mg)	Lipid (mg)	P:C	P:L
B. impatiens	25:1	475±58.5	5.46±0.90	0.56±0.11	1:87.01	9.84
	50:1	470±70.2	6.62±1.29	0.54±0.12	1:71.05	12.22
	75:1	344±46.7	3.84±0.90	0.37±0.15	1:89.55	10.49
	100:1	398±51.9	4.34±0.66	0.29±0.06	1:91.69	14.83
B. terrestris	25:1	199±29.5	2.74±0.41	0.25±0.05	1:72.41	10.83
	50:1	248±36.1	3.47±0.62	0.26±0.09	1:71.39	13.29
	75:1	264±65.4	4.09±1.32	0.32±0.13	1:64.61	12.98
	100:1	335±39.5	5.01±0.76	0.27±0.05	1:66.86	18.40

Consumption values are means±s.e.m. Each treatment was paired with a 5:1 P:L diet. Within each species, there were no statistical differences (Tukey-HSD pairwise comparisons) in total carbohydrate, protein or lipid consumed.

Journal of Experimental Biology (2016) 219, 3962-3970 doi:10.1242/jeb.140772

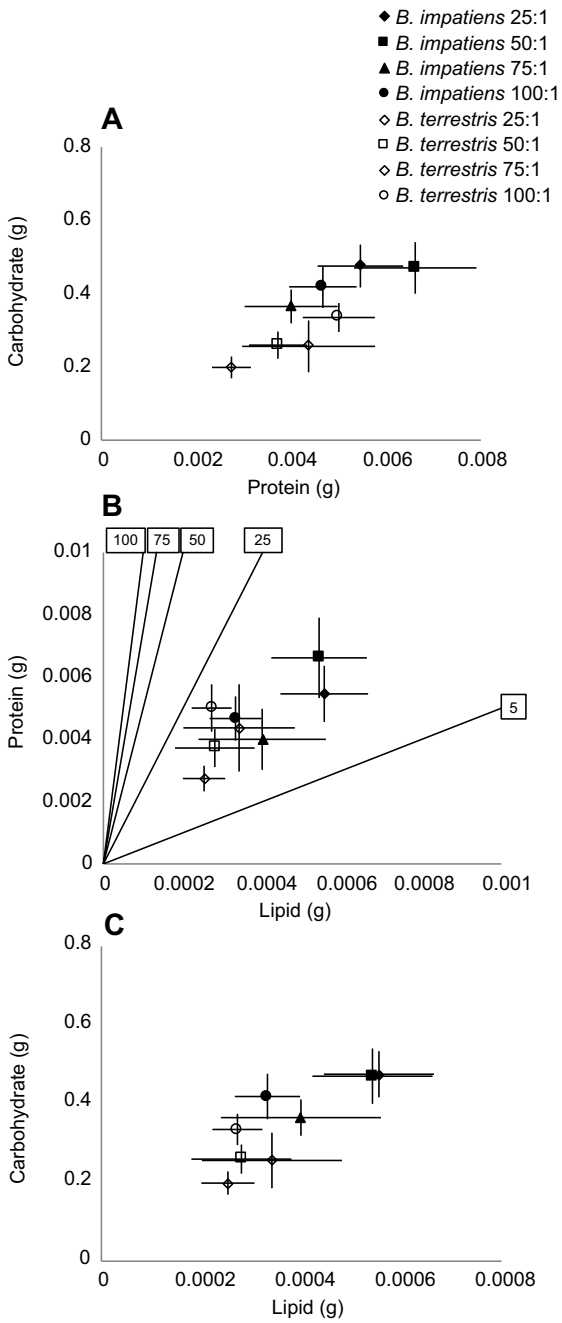

Fig. 5. Cumulative nutrient consumption of *B. impatiens* and *B. terrestris* foragers in paired P:L diets assay. Mean±s.e.m. cumulative consumption of nutrients by *B. impatiens* and *B. terrestris* foragers in paired P:L diets assay that survived for 7 days. Note that for both species there were no significant differences in carbohydrate, protein or lipid consumption across treatments. Treatments are represented by protein:lipid diet ratio (P:L) paired with 5:1 P:L diet. (A) Carbohydrate and protein. (B) Protein and lipid. Lines represent the different diet rails, emphasizing that across treatments all P:L intake targets lie within our expected 25:1–5:1 P:L range. (C) Carbohydrate and lipid. *Bombus impatiens*: $N_{25:1}$=16, $N_{50:1}$=16, $N_{75:1}$=12, $N_{100:1}$=16; *B. terrestris*: $N_{25:1}$=12, $N_{50:1}$=16, $N_{75:1}$=14, $N_{100:1}$=14.

paired diets, the bees indeed regulated to a particular P:L ratio and concentration of nutrients.

The exact mechanism underlying the toxicity of high-fat diet consumption is unclear. One possibility is a deficiency in protein intake, though this seems unlikely because adult bees can survive quite well on sugar diets alone (Brodschneider and Crailsheim, 2010; Paoli et al., 2014). Another possibility is that a high intracellular concentration of lipids is toxic; with too much fat in the diet, insufficient amounts could be converted into storage triacylglycerols or expelled from the body (Canavoso et al., 2001). The ratio of the essential fatty acids ω-6:ω-3 in our diets was 8:1. Excessive amount of ω-6 in diets (i.e. ω-3 deficiency) has been linked to chronic diseases in humans (Simopoulos, 2002, 2008), and impaired learning and physiology in honey bees (Arien et al., 2015). Moreover, high polyunsaturated fatty acids (including essential fatty acids) in the diet might lead to lipid peroxidation and cell damage, and cell membrane composition has been linked to the vast difference in maximum lifespan between honey bee queens (highly monounsaturated) and workers (highly polyunsaturated) (Haddad et al., 2007).

Although not the focal test of the study, bees consistently ate similar amounts of carbohydrates across all treatments in both the single and paired diets assays. The protein:carbohydrate ratio (P:C) intake target averaged 1:69 P:C for *B. terrestris* and 1:85 for *B. impatiens*. These intake targets are carbohydrate-biased as expected, but substantially lower than previously found for *B. terrestris* in studies that did not include lipid intake (Stabler et al., 2015). It might be that the energy otherwise obtained from carbohydrates (e.g. for flight) was metabolized from the lipids ingested in our study, resulting in reduced feeding from the sucrose-only solution (Canavoso et al., 2001).

The results of this study could provide insights into the nutritional ecology of foraging bees. First, the high requirement of carbohydrates for bumble bees is likely met by nectar foraging, which explains the attraction of bees to flowering species with high volumes and high sugar concentrations of nectar (Cnaani et al., 2006; Somme et al., 2015). Because carbohydrate concentrations in pollen are fairly low, bees seem to forage on pollen to meet their protein and lipid needs. Our results suggest that bumble bees forage to obtain pollen that allows them to achieve a dietary ratio of 12:1–14:1 P:L. Notably, in previous work, *B. impatiens* exponentially increased their foraging rates to plant species with a 5:1 P:L ratio; moreover, using assays with caged bees and nutritionally modified pollen, *B. impatiens* was most attracted to 5:1 and 10:1 P:L diets (Vaudo et al., 2016). These preferred diets matched the results from the current study, which found that bumble bee workers survive best on, and regulate their diets to, ∼10:1 P:L. Because the pollen P:L ratio in the previous work (Vaudo et al., 2016) had an upper limit of 5:1, it is unclear whether bumble bees can reach 10:1 P:L from pollen in the field. Even if the target P:L ratio cannot be met, the predisposition of bumble bees to prefer protein-biased pollen might explain host-plant preferences in natural environments (Cardoza et al., 2012; Hanley et al., 2008; Somme et al., 2015; Vaudo et al., 2016).

It must be noted that in the current study we evaluated feeding preferences of isolated bumble bee workers. It is unknown whether bumble bee foragers adjust their nutritional and foraging preferences depending on the colony needs, and specifically the presence of larvae (Hendriksma and Shafir, 2016). Information on pollen quality and its availability in the colony might be accessible to workers via pollen pots (Dornhaus and Chittka, 2005; Kitaoka and Nieh, 2009), allowing the colony to make informed foraging decisions. In our other studies, attraction of bumble bees to pollen with 5:1 and 10:1 P:L ratios remained intact for both bees foraging for colonies or foraging in cages (in the absence of brood), suggesting that these dietary preferences are conserved across a variety of scenarios (Vaudo et al., 2016).

Our study demonstrates that two bumble bee species, which occupy separate geographic ranges, regulate their protein-to-fat intake and exhibit similar intake targets, likely due to their relatedness, similar life histories and foraging behavior (Amsalem et al., 2015). Notably, their ability to regulate protein and lipids is more similar to arthropod predators than herbivores, perhaps because pollen is more nutritionally similar to prey (versus leaf tissue) with high protein and lipid concentrations (Jensen et al., 2011; Raubenheimer et al., 2007). Because bees are a monophyletic group evolved from predatory wasps (Danforth et al., 2013), it is likely that bees maintained their protein and lipid biases when making the transition to pollen feeding. There might be taxa-specific P:L intake targets across bee families, genera or species that could explain the patterns of foraging behavior and pollen preferences observed among host-plant species in field-based studies (Behmer and Joern, 2008). Knowing these particular intake targets could guide decisions for targeted habitat restoration protocols by matching nutritional intake targets of bee species to pollen quality of host-plant species (Vaudo et al., 2015).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
A.D.V.: Conceptualization, methodology, validation, formal analysis, investigation, writing – original draft preparation, writing – review and editing, visualization, funding acquisition; D.S.: Conceptualization, methodology, formal analysis, investigation; H.M.P.: Conceptualization, validation; J.F.T.: Conceptualization, validation, resources, writing – review and editing; C.M.G.: Conceptualization, validation, resources, writing – review and editing, funding acquisition; G.A.W.: Conceptualization, methodology, validation, resources, writing – review and editing, funding acquisition.

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