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

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Summary Statement

Bumble bees selectively feed among synthetic diets to acquire proteins and lipids ideal for survival. Optimal protein:lipid ratios are similar to the values of pollen from their preferred host-plant species.

Abstract

Bee population declines are linked to 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 their primary source of protein and lipids, but nutritional quality varies widely among host-plant species. Therefore, bees may be 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 *B. 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 overate protein on low fat diets and overate 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 P:L intake target of adult worker bumble bees was between 25:1-5:1, we presented workers from both species unbalanced but complementary paired diets to determine if 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.

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Introduction

Bee population declines are linked with 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 may 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.

Bees obtain their macronutrients (carbohydrates, proteins, and lipids) from floral nectar and pollen. Bees primarily obtain carbohydrates from nectar (Nicolson et al., 2007) to fuel energetically costly foraging efforts, and adults cannot survive without a continuous carbohydrate source (Brodschneider and Crailsheim, 2010). Bees obtain proteins and lipids from pollen. Differences in protein in bee diets can influence adult reproduction, physiology, and 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-dominant pollenkitt on the exterior of

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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 (pollen contains ~2-60% protein and ~2-20% lipid; (Roulston and Cane, 2000). Inequality of nutrients among plant species implies that bees may selectively forage for pollen to meet their nutritional demands. Generalist bee species, such as *Bombus terrestris* (Hymenoptera: Apidae) in Europe, North Africa, and the Middle East, and *B. impatiens* 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., 2014), 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., 2014). 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 their consumption of pollen diets depended on protein and lipid concentrations (Vaudo et al., 2016). This indicates that bees are sensitive to both protein and lipids in 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 eat pollen as well (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

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ovaries for male-egg laying (Amsalem et al., 2015; Tasei and Aupinel, 2008a). Note that in three-worker queenless microcolonies, workers ate between 0.4-0.9g of pollen in the five days prior to egg laying, which would average ~25-60mg/pollen/day by worker egg-layers (Tasei and Aupinel, 2008a; Tasei and Aupinel, 2008b).

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; Simpson and Raubenheimer, 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., 2009) and bumble bees (Stabler et al., 2015). Workers, especially foragers, have a high demand for carbohydrates, as reflected in their measured intake targets (for bumble bees, this is ~1:150 protein:carbohydrate or 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., 2009; 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., 2009).

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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 using the GF of 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* regulated its diet by eating flies that complemented a previous diet higher in protein or fat (Mayntz et al., 2005), and overate 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. Then, using the results of the first experiments to select appropriate diets, we presented *B. terrestris* and *B. impatiens* individuals 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 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

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provide insights into the ability of bumble bees to regulate lipids in their diet, suggesting nutritional quality may drive pollen foraging preferences.

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 approximately 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-hr 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 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/treatment, 4 colonies) were given access to food tubes containing 0.5 M sucrose solution or 0.5 M sucrose solution containing a specific

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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 the 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 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 hours under low heat. Liquid diets were used because they are easy for the bees to ingest and allow accurate measurement of consumption.

Experiments lasted seven days, and we replaced each food tube daily. We weighed food tubes each day prior to placement in the cage and 24 hr later. Cages with three tubes of each diet (replaced daily) with 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, lipid) consumed from the total mass consumed from each

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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 seven days.

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 "Results-Single P:L diet assay"), we measured survival and nutrient consumption of *B. impatiens* and *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/treatment; 2 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 seven days (see "Single P:L diet assay"). Prior to placement in cages, we cold anaesthetized and weighed foragers to use their weight as a covariate in data analyses to control for effects of size on diet consumption (note thorax width and bee weight are correlated [Stabler et al., 2015], and we measured thorax width in the "Single P:L diet assay").

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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 of adding lipid to the diet on bee survival. 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 2-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 seven 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 *Bombus impatiens* were analyzed separately. We analyzed differences in survival among treatments with the Kaplan-Meier test (because there was no reference group as

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above for Cox-regression). To determine daily differences in mass of diets consumed among treatments, we conducted 2-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 weight as covariates. Note that bee weight was used as a measure of size for this assay while thorax width was used in the *"Single P:L diet assay"*. These are correlated metrics of bee size used as covariates for consumption/bee (Stabler et al., 2015). Finally, for bees that survived all seven days, we analyzed cumulative nutrient consumption among treatments with MANCOVA with *post-hoc* Tukey-HSD pairwise comparisons with nutrient (carbohydrate, protein, or lipid) as the dependent variable and colony and bee weight 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).

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Results

Single P:L diet assay

For seven 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 "protein only" treatment ate more each day than bees on the high fat 1:5 P:L treatment at $P < 0.05$ (Figure 1). Bees differed in the relative amounts of each diet (treatment diet versus sucrose only) consumed (treatment x solution; $F_{7,1321}$ = 16.0, $P < 0.001$) (Figure 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; Figure 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 seven 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; Figure 2). Though there were no differences in cumulative carbohydrates consumed

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across treatments ($F_{7,59}$ = 1.13, P = 0.36; Figure 2a,c), bees on different diets consumed significantly different amounts of cumulative protein and lipids over seven 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; Figure 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, Figure 2b,c). Furthermore, bumble bee foragers consumed on average ~3.5mg protein on 1:1, 5:1, 10:1 and 25:1 P:L diets, while consuming ~5.1mg 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 (Figure 2b). These data also indicate that *B. terrestris* foragers regulated their protein intake eating similar amounts of proteins (~4.0mg) except on the highest lipid diets of 1:5 and 1:10 (~0.6mg).

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 seven days (Table 3). Although bees in the high fat treatment (1:5 P:L) appeared 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 (Figure 1, Figure 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

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living and dead bees ate equal amounts of carbohydrates (t_{14} = 0.64, P = 0.27; Figure 4). These data suggest that high lipid consumption leads to toxicity and increased mortality.

Bombus terrestris foragers 1) overate 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 bumble bees' P:L intake target 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*.

Paired P:L diets assay

For seven days, we fed *Bombus 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*: $F_{2,1178}$ = 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; Figure 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 (Figure S1).

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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: *B. impatiens*: MANCOVA: $F_{9,122}$ = 1.35, P = 0.22; *B. terrestris*: MANCOVA: $F_{9,110}$ = 1.07, P = 0.39; Table 4, Figure 5, Figure 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, Figure 5, Figure 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, Figure 5,Figure S2). Both bee species survived equally well on the various diets (*B. impatiens*: χ^2 = 3.98, df = 3, P = 0.26; *B. terrestris*: χ^2 = 0.39, df = 3, P = 0.94; Figure S3).

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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, Figure 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 bees 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 appears more similar to predaceous arthropods than herbivorous ones. *Manduca sexta* caterpillars, within a similar design as 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. Both 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 in 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 may also overeat protein to reach their lipid intake; indeed, lipid intake did not differ across the groups fed 50:1,

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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 *A. dorsale*, the predatory ground beetle, which stopped eating when it reached its lipid intake target in high fat diets (Raubenheimer et al., 2007), *B. terrestris* overate 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 may have led to increased mortality. For example, bees survived when they ate less of the high fat diet 1:10 P:L (Figure 4). And 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 (Figure 1-3). Thus, it appears that the surviving bees were able to eat enough to meet their nutritional needs, sense the toxicity of the diet, and cease feeding, while 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 may have been physiological differences associated with age, social status, or behavioral task. Additionally, 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).

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Although feeding behavior may 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 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 high intracellular concentrations 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; Simopoulos, 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 may 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).

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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 significantly lower than previously found for *B. terrestris* in studies that did not include lipid intake (Stabler et al., 2015). It may 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 may 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., 2014). Because carbohydrate concentrations in pollen are fairly low, bees appear 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 the plant species with the 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, approximately 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

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protein-biased pollen may explain host-plant preferences in natural environments (Cardoza et al., 2012; Hanley et al., 2008; Somme et al., 2014; 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 presence of larvae (Hendriksma and Shafir, 2016). Information on pollen quality and its availability in the colony may be accessible to workers via pollen pots (Dornhaus and Chittka, 2005; Kitaoka and Nieh, 2008) 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 demonstrated 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 may 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

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field-based studies (Behmer and Joern, 2008). Knowing these particular intake targets can 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).

List of Symbols and Abbreviations

GF – Geometric framework for nutrition

P:C – Protein to carbohydrate ratio

P:L – Protein to lipid ratio

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Competing Interests

No competing interests declared.

Author Contributions

Vaudo: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft Preparation, Writing – Review & Editing, Visualization, Funding Acquisition

Stabler: Conceptualization, Methodology, Formal Analysis, Investigation

Patch: Conceptualization, Validation

Tooker: Conceptualization, Validation, Resources, Writing – Review & Editing

Grozinger: Conceptualization, Validation, Resources, Writing – Review & Editing, Funding Acquisition

Wright: Conceptualization, Methodology, Validation, Resources, Writing – Review & Editing, Funding Acquisition

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Figures

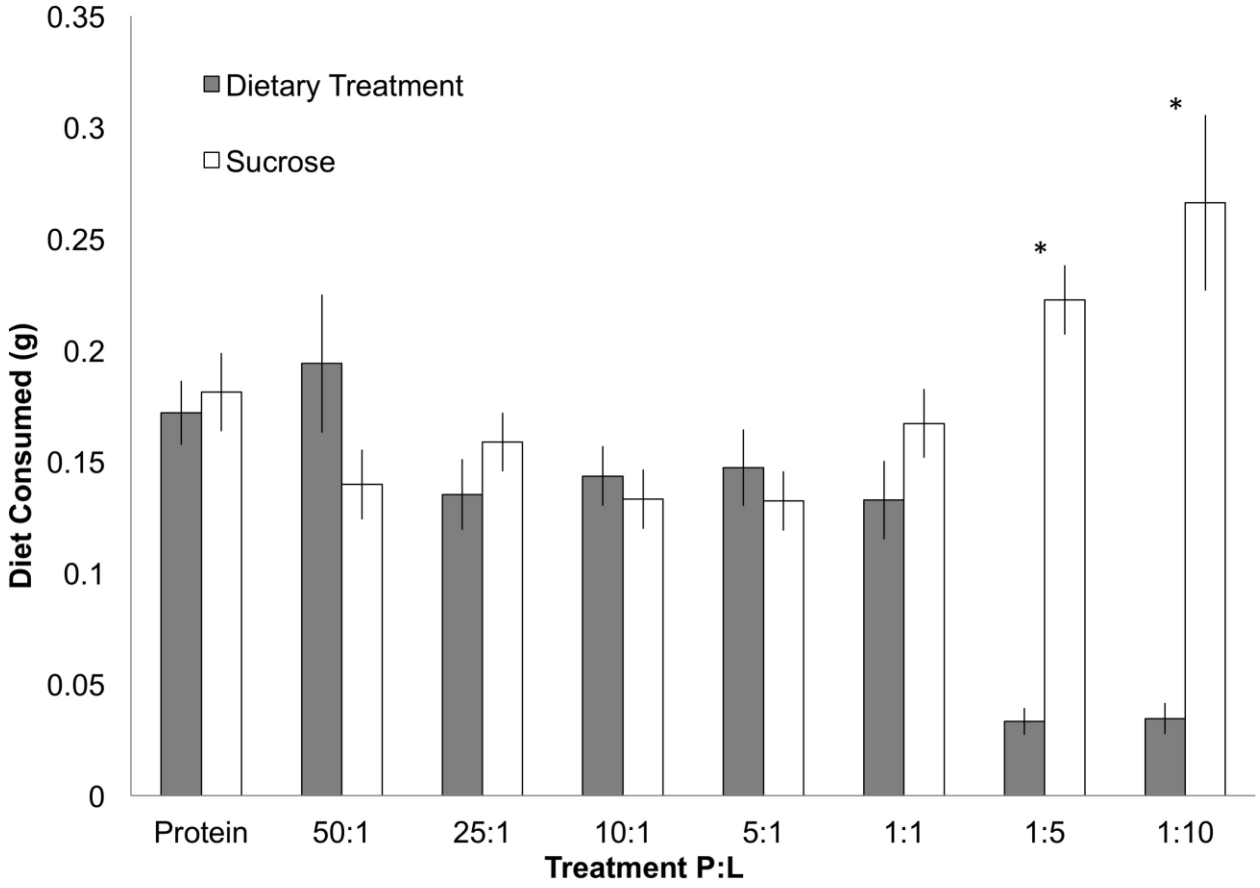

Figure 1. Mean (± SE) daily consumption of diets across treatments for *B. terrestris* foragers in "Single P:L diet assay." Treatments are represented by their protein:lipid (P:L) treatment diet ratio, including protein-only diets. Diets are represented as sucrose-only and diet associated with each treatment. Asterisks represent significant differences ($P < 0.05$) in diet consumed within treatment (N = 15 bees/treatment).

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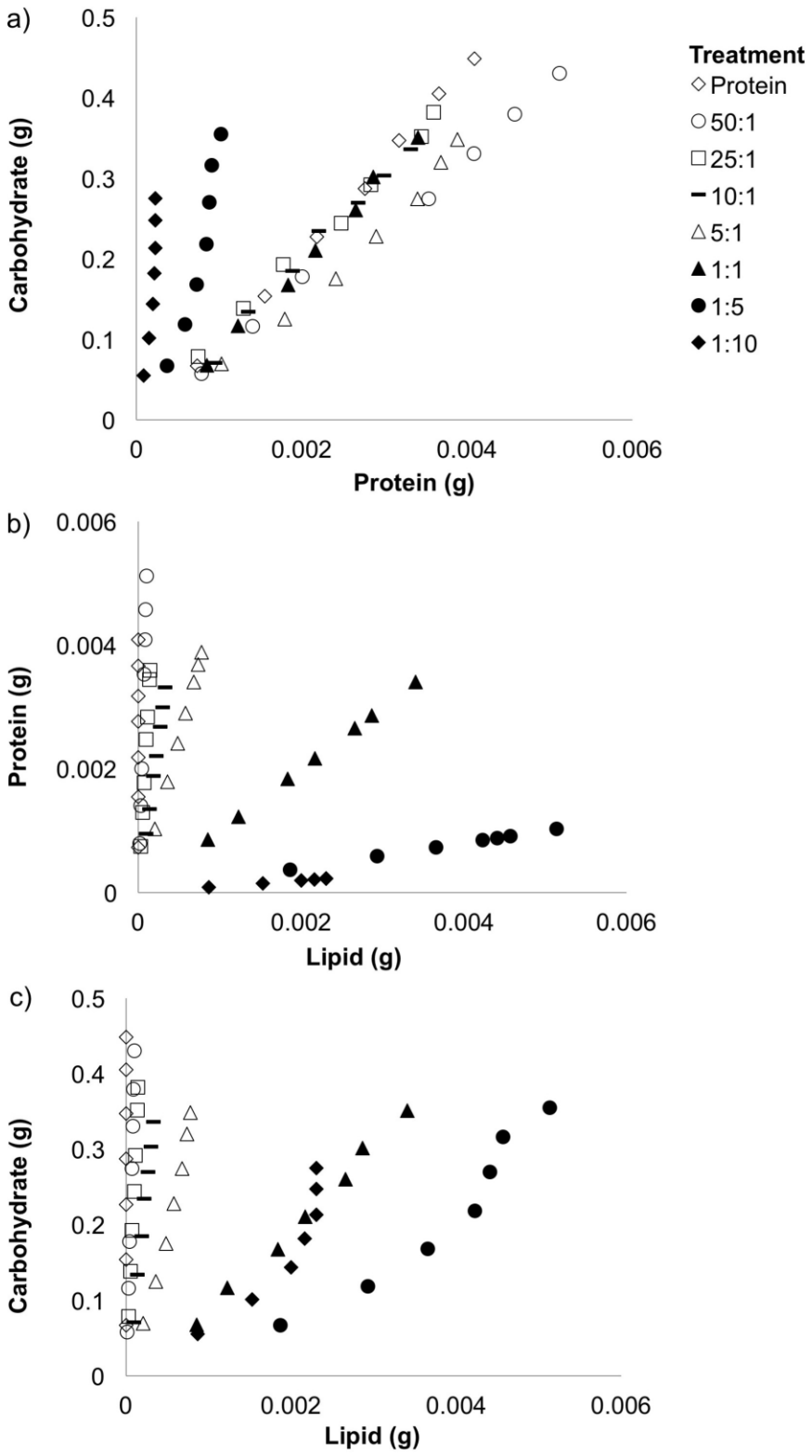
a)
Carbohydrate (g)
Protein (g)
0
0.1
0.2
0.3
0.4
0.5
0.002
0.004
0.006
Treatment
Protein
50:1
25:1
10:1
5:1
1:1
1:5
1:10
b)
Protein (g)
Lipid (g)
c)
Carbohydrate (g)
Lipid (g)

Figure 2. Nutritional arrays of *B. terrestris* foragers surviving seven 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 seven days forming daily trajectories. a) carbohydrate and protein array, b) protein and lipid array, 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).

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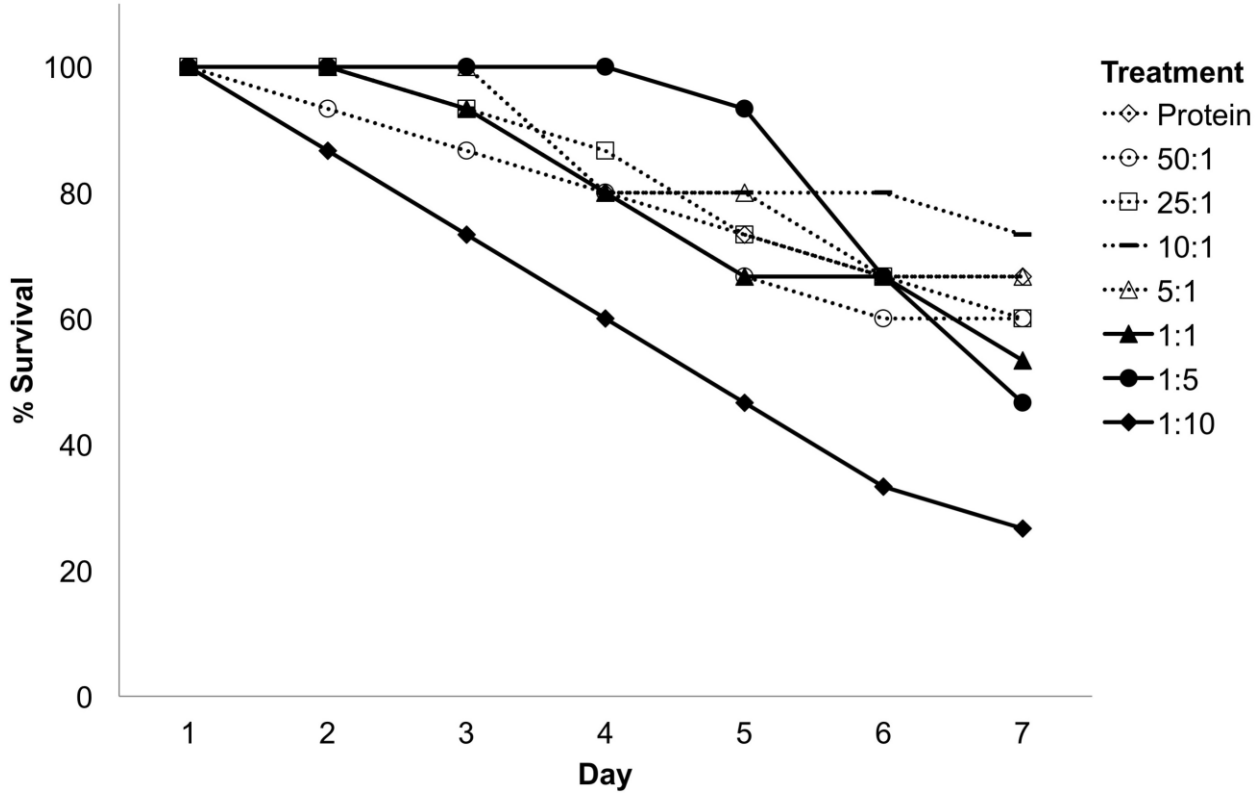

Figure 3. Survival curve of *B. terrestris* foragers in "Single P:L diet assay." Treatments are represented by their protein:lipid (P:L) treatment diet ratio, including protein-only diet. Note that mortality increased as the lipid content of the diets increased (N = 15 bees/treatment).

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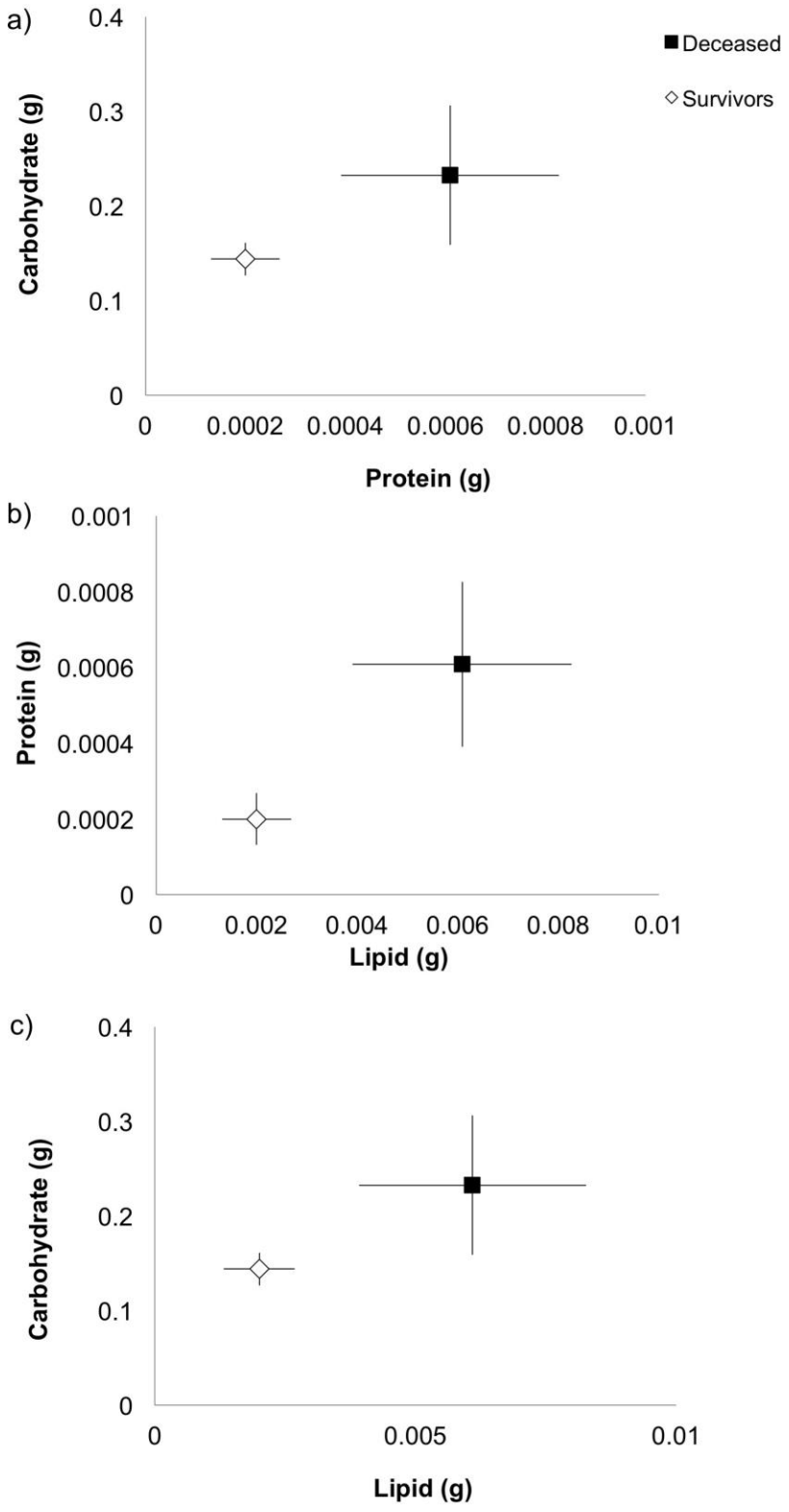
a)
Deceased
Survivors
Carbohydrate (g)
0.4
0.3
0.2
0.1
0
0
0.0002
0.0004
0.0006
0.0008
0.001
Protein (g)
b)
Protein (g)
0.001
0.0008
0.0006
0.0004
0.0002
0
0
0.002
0.004
0.006
0.008
0.01
Lipid (g)
c)
Carbohydrate (g)
0.4
0.3
0.2
0.1
0
0
0.005
0.01
Lipid (g)

Figure 4. Mean (± SE) cumulative consumption of nutrients by deceased (N = 11) and surviving (N = 4) *B. terrestris* foragers in 1:10 P:L treatment on Day 3 of "Single P:L diet assay": a) carbohydrate and protein, b) protein and lipid, c) carbohydrate and protein. Note that surviving bees ate significantly less protein and lipid than the deceased bees.

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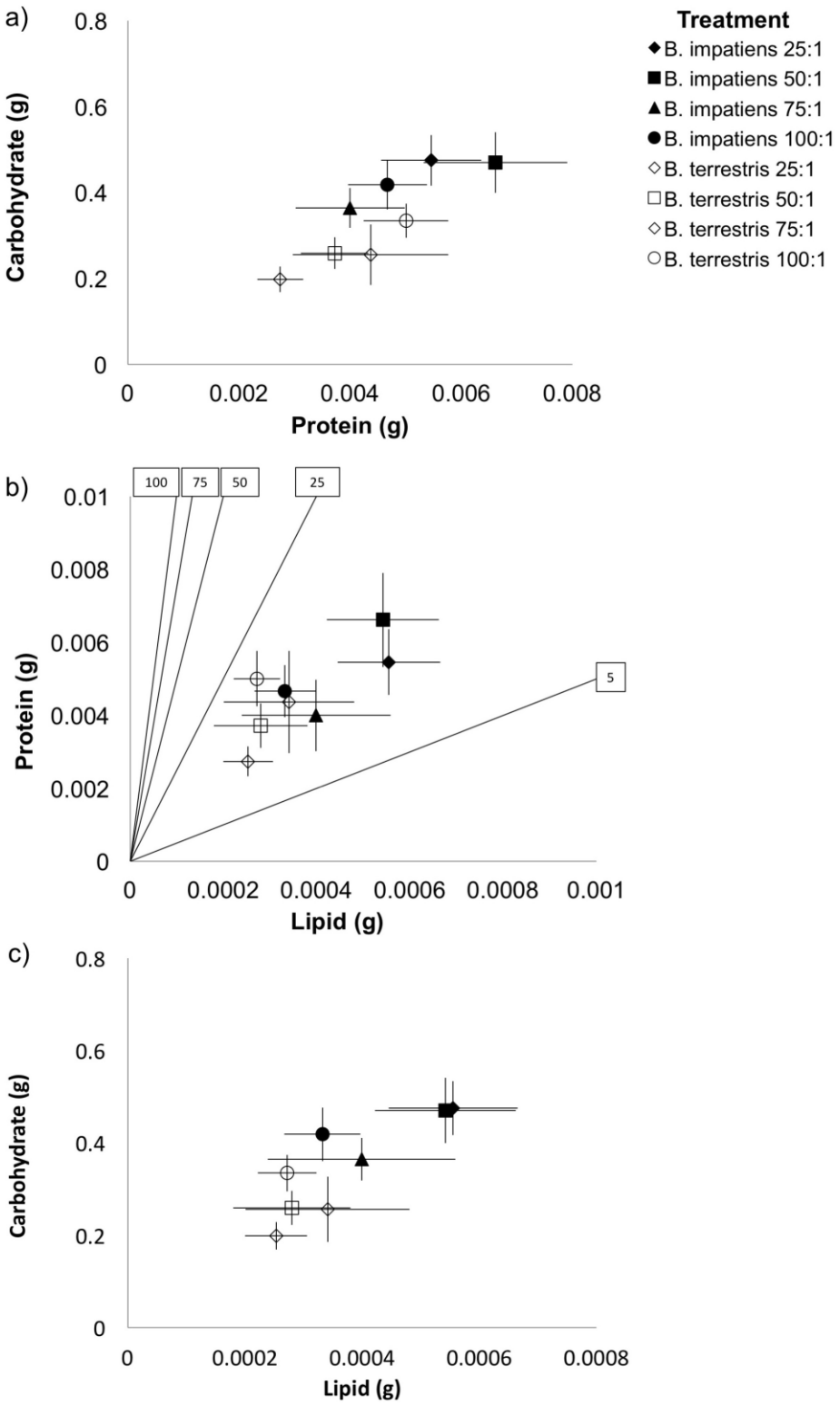
a)
Carbohydrate (g)
0.8
0.6
0.4
0.2
0
0
0.002
0.004
0.006
0.008
Protein (g)
Treatment
B. impatiens 25:1
B. impatiens 50:1
B. impatiens 75:1
B. impatiens 100:1
B. terrestris 25:1
B. terrestris 50:1
B. terrestris 75:1
B. terrestris 100:1
b)
Protein (g)
0.01
0.008
0.006
0.004
0.002
0
100
75
50
25
5
0
0.0002
0.0004
0.0006
0.0008
0.001
Lipid (g)
c)
Carbohydrate (g)
0.8
0.6
0.4
0.2
0
0
0.0002
0.0004
0.0006
0.0008
Lipid (g)

Figure 5. Mean (± SE) cumulative consumption nutrients of *B. impatiens* and *B. terrestris* foragers in "Paired P:L diets assay" that survived for seven days. Note 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 protein (*B. impatiens*: $N_{25:1} = 16$, $N_{50:1} = 16$, $N_{75:1} = 12$, $N_{100:1} = 16$; *B. terrestris*: $N_{25:1} = 12$, $N_{50:1} = 16$, $N_{75:1} = 14$, $N_{100:1} = 14$).

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Table 1. Diet recipes. 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.

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	171g	17.1g	17.1g	17.1g	17.1g	17.1g	17.1g	17.1g	17.1g	17.1g	17.1g
Casein	-	0.342g	0.342g	0.342g	0.342g	0.342g	0.342g	0.342g	0.342g	0.342g	0.342g
Lecithin	-	-	0.00342g	0.00456g	0.00685g	0.0137g	0.0342g	0.0685g	0.342g	1.71g	3.42g
H_2O	1L	100mL	100mL	100mL	100mL	100mL	100mL	100mL	100mL	100mL	100mL

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Table 2. Mean (± SE) daily consumption (mg) of nutrients for *B. terrestris* foragers in "Single P:L diet assay." 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 ($P < 0.05$).

Treatment	Carbohydrate	Protein	Lipid
1:10	50 ± 7 ab	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 ab	0.44 ± 0.06 a	0.44 ± 0.06 bc
5:1	47 ± 3 ab	0.50 ± 0.06 a	0.11 ± 0.012 cd
10:1	47 ± 3 ab	0.49 ± 0.05 a	0.05 ± 0.005 d
25:1	50 ± 3 ab	0.47 ± 0.05 a	0.02 ± 0.002 d
50:1	57 ± 5 ab	0.66 ± 0.11 a	0.01 ± 0.002 d
Protein-only	60 ± 4 a	0.60 ± 0.05 a	-

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Table 3. Cox – regression of survival for *B. terrestris* foragers in "Single P:L diet assay." 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: $\chi^2 = 10.52$, df = 7, p = 0.161

							95.0% CI for Exp(B)	
Treatment	**B**	**SE**	**χ^2**	**df**	**Sig.**	**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

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Table 4. Consumption (g; mean ± SE) 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 seven days. Each treatment was paired with a 5:1 P:L diet. Within each species, there were no statistical differences in total carbohydrate, protein, or lipid consumed.

	Treatment	**Carbohydrate**	**Protein**	**Lipid**	**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

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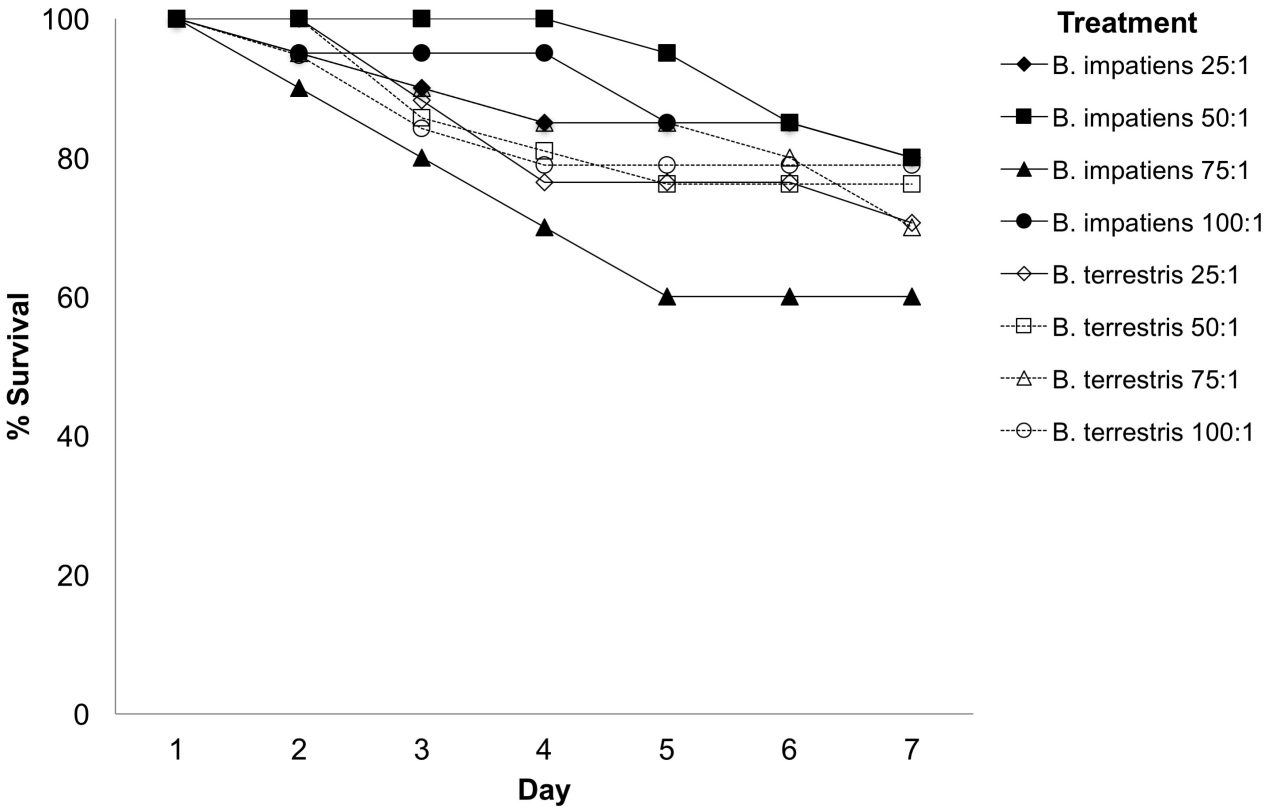

Supplementary Figure 1. Mean (± SE) daily consumption of diets across treatments for a) *B. impatiens* and b) *B. terrestris* foragers in "Paired P:L diets assay." Diets are represented as 5:1 P:L, sucrose-only, and the treatment P:L diet (25:1, 50:1, 75:1, and 100:1). Bars marked with different letters are statistically different ($P < 0.05$) within treatment (N = 20 bees/treatment).

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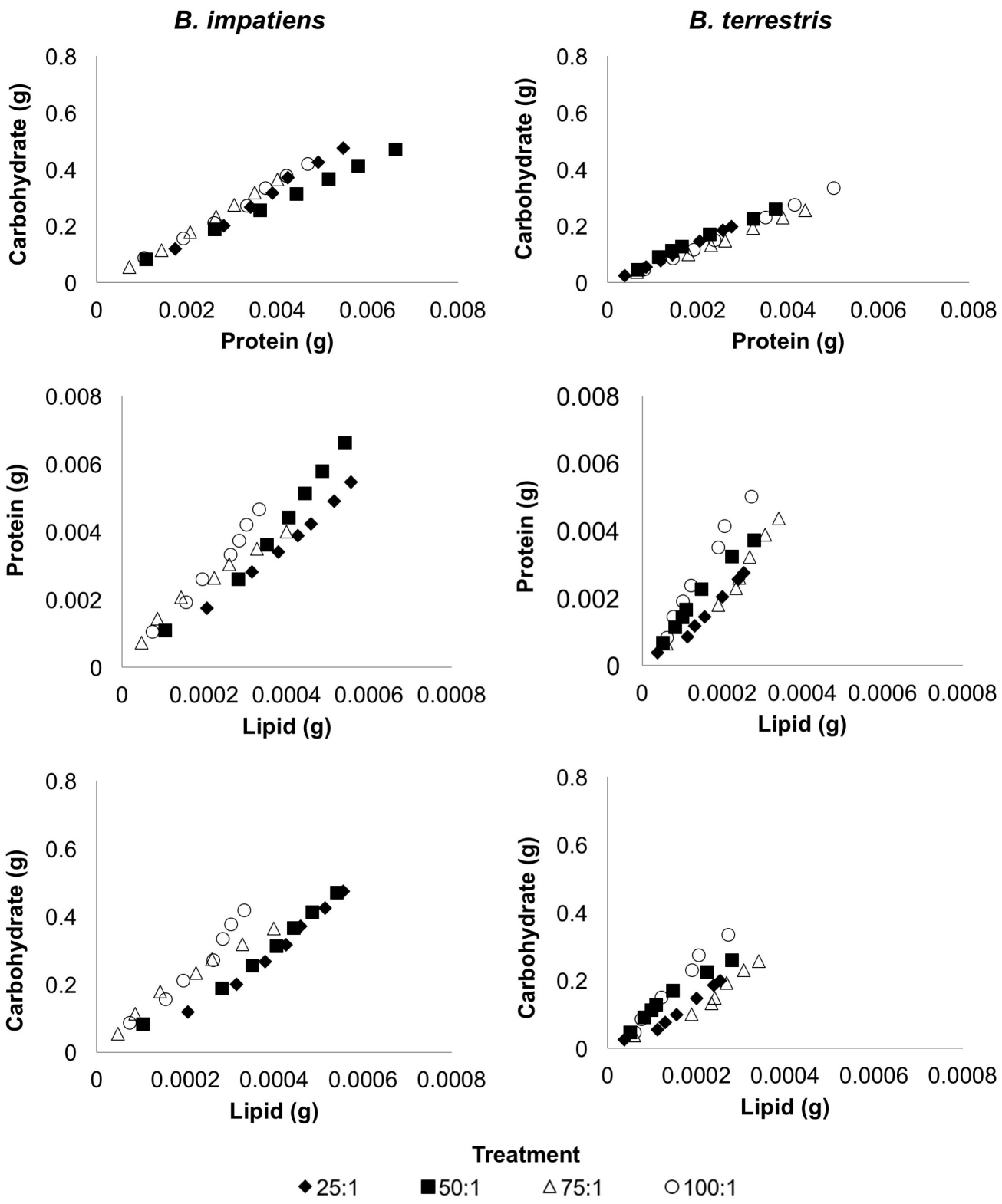

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Supplementary Figure 2. Daily trajectories of *B. impatiens* (a-c) and *B. terrestris* (d-f) in "Paired P:L diets assay." Treatments are represented by their protein:lipid diet ratio (P:L) paired with 5:1 P:L diet. Markers within each diet represent mean cumulative consumption of each nutrient for each successive day up to seven days: a,d) carbohydrate and protein trajectories, b,e) protein and lipid trajectories, c,f) carbohydrate and lipid trajectories (*B. 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).

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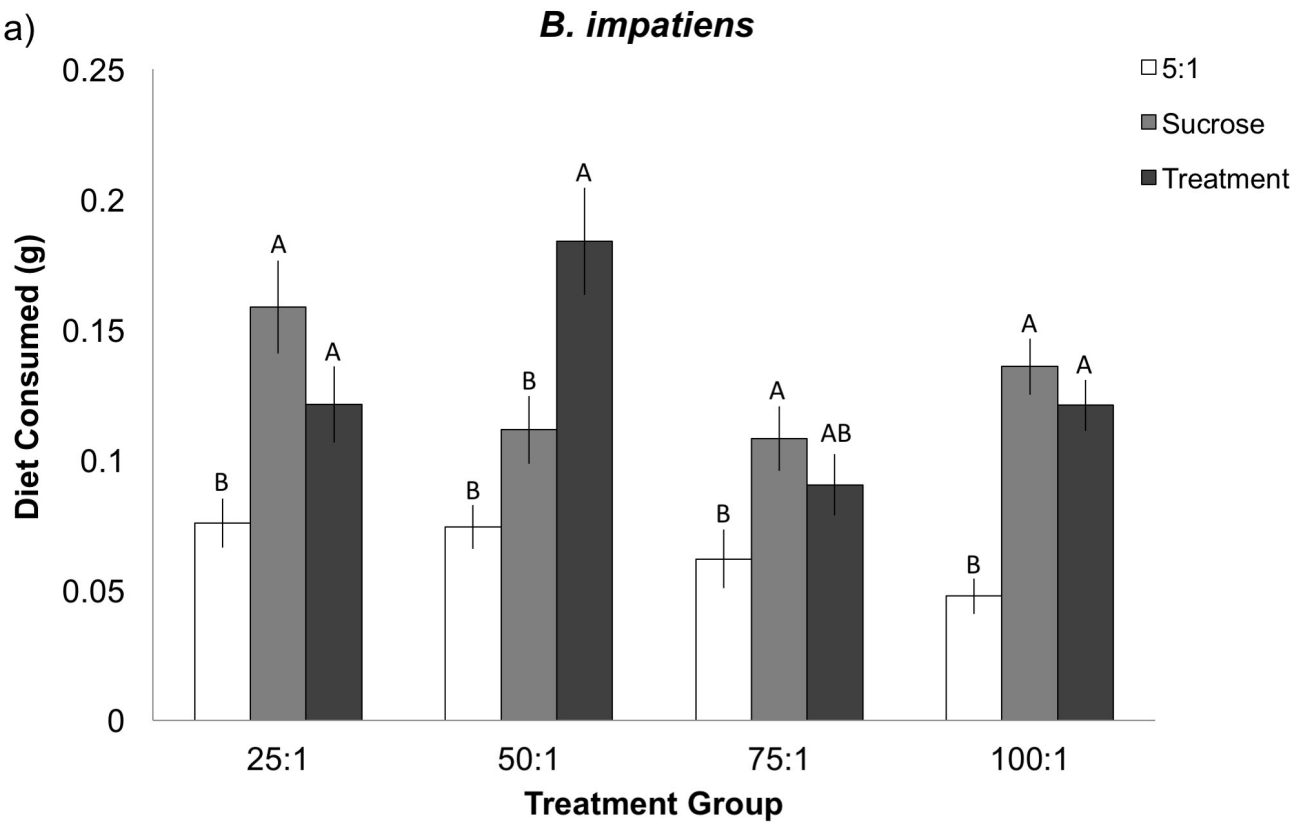

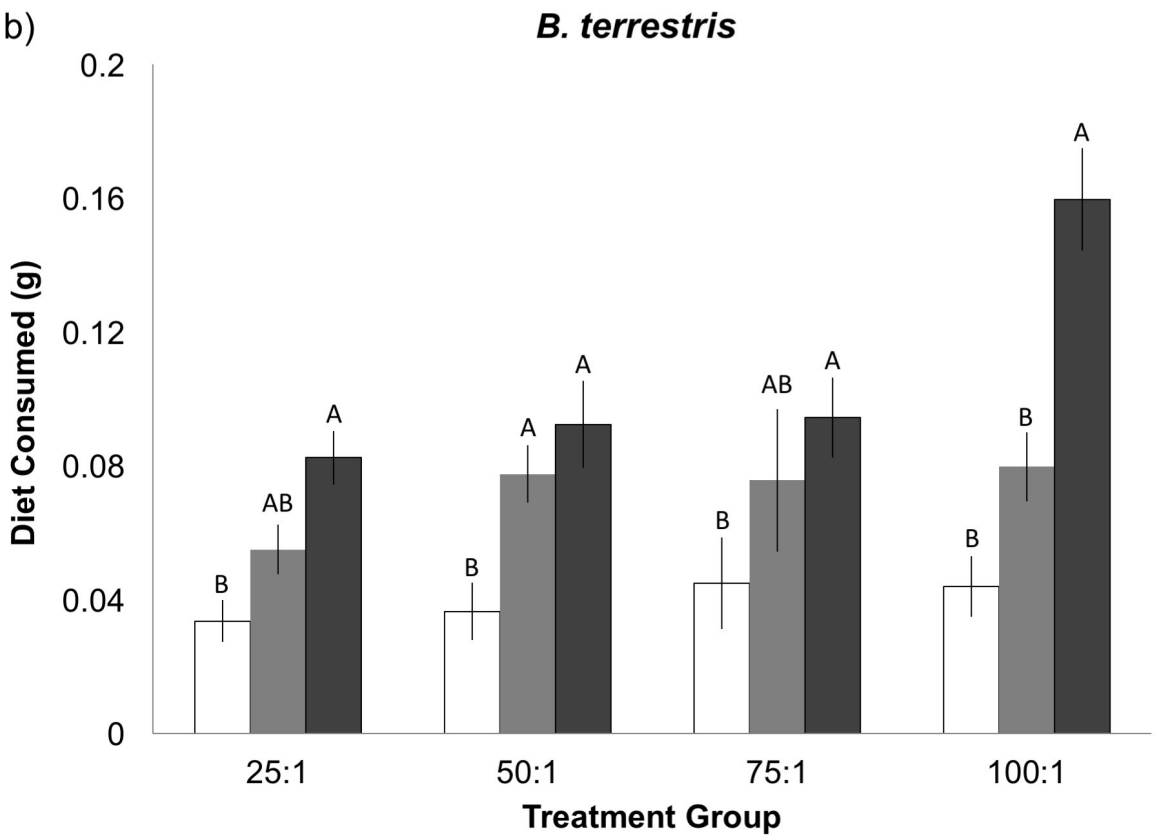

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Supplementary Figure 3. Survival curve of *B. impatiens* and *B. terrestris* foragers in "Paired P:L diets assay." Treatments are represented by their species and protein:lipid diet ratio (P:L) paired with 5:1 P:L diet (N = 20 bees/treatment).

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