Diet and nutritional status during early adult life have immediate and persistent effects on queen bumble bees

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Many insects sequester nutrients during developmentally programmed periods, which they metabolize during subsequent life history stages. During these periods, failure to store adequate nutrients can have persistent effects on fitness. Here, we examined a critical but under-studied nutrient storage period in queen bumble bees: the first days of adult life, which are followed by a diapause period typically coinciding with winter. We experimentally manipulated availability of pollen (the primary dietary source of lipids and protein) and the sugar concentration of artificial nectar (the primary source of carbohydrates) for laboratory-reared queens during this period and examined three nutritional phenomena: (i) diet impacts on nutritional status, (ii) the timescale upon which nutrient sequestration occurs and (iii) the fitness consequences of nutrient sequestration, specifically related to survival across the life cycle. We found evidence that pollen and nectar starvation negatively impact lipid storage, whereas nectar sugar concentration impacts stored carbohydrates. The majority of nutrients were stored during the first ~3 days of adult life. Nutrients derived from pollen during this period appear to be more critical for surviving earlier life stages, whereas nutrients sequestered from nectar become more important for surviving the diapause and post-diapause periods. Negative impacts of a poor diet during early life persisted in our experiment, even when pollen and a relatively high (50%) nectar sugar concentration were provided post-diapause. Based on these findings, we posit that the nutritional environment during the early adult life of queens has both immediate and persistent impacts on fitness. These findings underscore the importance of examining effects of stage-specific nutritional limitations on physiology and life history traits in this social insect group. Moreover, the findings may shed light on how declining food resources are contributing to the decline of wild bumble bee populations.

Key words: Bees, diapause, nutrition

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Introduction

Bumble bees (genus *Bombus*, family Apidae) are a group of largely social bees that are found most often in temperate, alpine and subalpine regions. They play important roles as pollinators in many natural ecosystems (Goulson, 2010; Corbet et al., 1991) and are often considered keystone species in plant–pollinator networks because of their tendency to visit both rare and abundant plant species (Burkle et al., 2013; Goulson et al., 2008b; Memmott et al., 2004). Bumble bees are also among the most effective pollinators of several agricultural crops, such as blueberry and cranberry (Kremen et al., 2002; Stubbins and Drummond, 2001), and greenhouse crops, such as tomatoes (Velthuis and Van Doorn, 2006). Their effectiveness is due in part to their relatively large body size and because they are capable of buzz pollination, a specialized sonication behaviour that causes some plants to release pollen from poricidal anthers (De Luca and Vallejo-Marín, 2013).

Despite their ecological and economic importance, there is increasing evidence that many bumble bee populations worldwide are threatened, with declines in abundance and range reductions detected in a number of species (Goulson et al., 2008a; Williams and Osborne, 2009). One of the key drivers of bumble bee decline appears to be nutritional stress, which stems from widespread loss of flower-rich foraging habitat due to agricultural intensification and other anthropogenic processes (Goulson et al., 2015; Woodard, 2017).

Temporal and spatial population dynamics in bumble bees are strongly influenced by characteristics of the queen caste in particular (relative to worker or male traits). Given that workers are unable to mate or lay fertilized eggs, queens are crucial for overall colony reproduction. Temperate, social bumble bee species also have an annual colony cycle wherein queen overwintering survival and subsequent reproductive capacity are likely major population dynamic bottlenecks. New queens overwinter for a period of several months in a diapause state (Goulson, 2010; Sladen, 1912). During this pre-diapause period, queens undergo a suite of physiological changes in preparation for diapause, perhaps the most dramatic of which is the accumulation of copious amounts of stored glycogen (but see Votavová et al., 2015) and also lipids in adipocytes of the fat body (Alford, 1969b; Fliszkiewicz and Wilkaniec, 2007; Röseler and Röseler, 1986; Votavová et al., 2015). Workers and male bumble bees do not undergo these stereotypical changes in nutrient levels (Alford 1969b; Röseler and Röseler, 1986). As is true of the overwhelming majority of bee species, reproductive females obtain lipids and protein primarily from pollen (reviewed in Roulston and Cane, 2000), whereas glycogen is produced from sugars primarily obtained from nectar (largely sucrose, with lesser amounts of glucose and fructose; reviewed in Baker and Baker, 1983). Queen bumble bees metabolize most of their stored lipids and glycogen during diapause (Alford, 1969a, 1969b; Holm, 1972; Přidal and Hofbauer, 1996). Thus, access to pollen and nectar during the pre-diapause period likely plays a key role in queen overwintering survival. Despite the importance of queens for population dynamics, and increasing evidence that nutritional stress is a primary driver of bumble bee declines (Goulson et al., 2015; Woodard, 2017), examinations of diet impacts on queen nutrient storage and fitness have not yet been performed.

To address this knowledge gap, we investigated how diet impacts macronutrient storage (lipids, glycogen and protein), the timescale upon which this occurs and the consequences of early-life nutrition in queens of the North American common eastern bumble bee, *Bombus impatiens*. This species is highly amenable to laboratory research, making it a system where phenomena that are difficult to study in wild bumble bees, including during the various solitary life history stages in queens, can be explored in the laboratory under controlled conditions. Our first objective was to determine how diet, specifically the presence or absence of pollen and the sugar concentration of nectar, impacts queen nutritional status during the pre-diapause period. Given the key nutrients queens obtain from each of these two main food resources, we predicted that queens without access to pollen would exhibit reduced lipid levels, whereas queens deprived of nectar-derived sugar would have reduced stored carbohydrate (i.e. glycogen) amounts. Our second objective was to determine the timescale upon which nutrient sequestration occurs, using weight gain across the period as an indirect but repeatable proxy for nutrient storage. Based on previous work by Alford (1969b), we predicted that the majority of nutrient storage would occur during the first few days of adult life, although these past analyses were performed in queens of different bumble bee species and did not examine how sequestration is impacted by diet. Our third objective was to examine the consequences of diet on queen survival during the nutrient sequestration period and also during the subsequent diapause and post-diapause periods. Here, we predicted that mortality might be particularly high during the diapause period, during which queens are known to metabolize the majority of stored nutrients.
Table 1: Diet treatment groups

| Treatment group          | Pollen | Nectar    |
|--------------------------|--------|-----------|
| Control diet             | Control| Yes 50%   |
| Pollen manipulation      | Pollen-starved | No 50%   |
| Nectar manipulations     | Nectar-starved | Yes 0%   |
| Low-concentration nectar | Low-concentration nectar | Yes 25% |
| High-concentration nectar| High-concentration nectar | Yes 75% |

*Column entitled ‘Pollen’ refers to whether queens were provided with pollen (‘Yes’) or not (‘No’). For nectar manipulation treatment groups, percentages correspond to nectar sucrose concentration (w/v).

Materials and methods

Bee rearing and diet treatments

All queens originated from mature colonies (with > 50 workers and queenright) supplied by Koppert Biological Systems (Howell, MI). Colonies were maintained at 23 ± 3°C, 50 ± 5% RH, and under darkness or dim red light, and supplied with honey bee-collected pollen (stored frozen; obtained from Brushy Mountain Bee Farm, Moravian Falls, NC) and nectar syrup (supplied by Koppert Biological Systems) provided ad libitum. Newly eclosed or ‘callow’ adult queens (age < 24 h; hereafter, referred to as age ‘0 day’) were identified by their characteristic silvery appearance, removed from their natal colonies and individually housed in small plastic containers (6 × 3 × 3 in) in a room maintained at 28 ± 2.5°C and 60 ± 5% RH.

Queens were subjected to one of five diet treatments varying in either pollen availability (starvation or provided ad libitum) or nectar with varying sugar concentrations (Table 1). The ‘control’ treatment was given a mixture of honey bee-collected pollen (of mixed origin, consistent with the generalist diet of bumble bees) and 50% (w/v) sucrose solution and was used as a control for both pollen and nectar diet manipulations. Many floral nectars contain sucrose concentrations greater than 50% but less than 75% (Chalcoff et al., 2006); our experimental nectar concentrations were designed to either completely remove nectar-derived sugar from the diet (0% w/v sucrose solution; ‘nectar-starved’) or mimic nectars with low but realistic (25% w/v sucrose solution; ‘low concentration’), normal (50% w/v sucrose solution; ‘control’) or abnormally high (75% w/v sucrose solution; ‘high concentration’) sugar concentrations. Honey bee-collected pollen does contain some sugar that is added to pollen as it is collected (Roulston and Cane, 2000), and so nectar-starved queens in our experiment had some sugar in their diets. The diet treatment administration period was from ages 0 to 11 days, in accordance with the approximate natural length of the post-eclosion, pre-diapause nutrient sequestration period in queen bumble bees (Alford, 1969b). All diet treatments were administered ad libitum, and pollen was mixed with a sucrose solution equal in concentration to the solution provided in the liquid feeder (for example, pollen was mixed with water in the nectar-starved group). Throughout the feeding treatment period, fresh pollen and nectar were provided every 1–2 days and queens were checked daily for mortality.

Nutritional status

To examine effects of diet on nutrient sequestration, a set of queens originating from 14 source colonies (n = 9–25 per treatment group) were collected after the diet treatment administration period (at age 11 days), immediately stored at −80°C, then used for spectrophotometric quantification of crude, whole abdomen lipids, protein and stored carbohydrate (glycogen). Whole abdomens were used for this analysis. This body segment contains the majority of the fat body (Alford, 1969b), in addition to other tissues such as the ovaries and digestive tract. We performed a separate analysis to determine whether short-term (24-h) starvation prior to collection and/or removal of the gut (including crop) and venom sac prior to tissue homogenization influenced results of the nutritional analyses; these methods did not significantly influence these results and so were not performed in the study (see Supplementary Information). Abdomens were homogenized in 1500 μL of water with steel beads added and agitated for 5 min at maximum frequency using a Qiagen TissueLyser, then centrifuged at 14000×g for 5 min. From the homogenized sample, the liquid fraction was divided and used for protein and glycogen assays; the solid pellet was reserved for lipid assays. Because of their high concentration, aliquots of the homogenized sample were used for glycogen and protein quantitation, as described below. For all samples, quantitation was performed on a Varioskan LUX (Thermo Fisher) microplate reader using the SkanIt Software (v. 4.1) and samples were prepared in a serial dilution, measured in triplicate, averaged and multiplied by the dilution factor to estimate macronutrient amounts (mg) per mL of sample.

Abdominal lipid concentrations were quantified using a sulfo-phospho-vanillin assay adapted from Cheng et al. (2011) and McMahon et al. (2013) and used previously in lipid studies in bees (Toth et al., 2005) and wasps (Daugherty et al., 2011). One millilitre of 2:1 chloroform:methanol solution was added to the solid fraction of homogenized tissue in a 2-mL tube and vortexed thoroughly to dissolve lipids. Samples were centrifuged for 2 min at 14000×g, and the liquid fraction was aliquoted into a 5-mL glass vial with a Teflon-lined cap for storage at −80°C. An additional 3 mL of 2:1 chloroform:methanol was added to the sample, and then 11, 8, 5 and 2 μL of the bottom chloroform layer was added in triplicate to wells in the microplate. Chloroform was evaporated at 90°C, then 100 μL of concentrated sulphuric
acid was added to each well and incubated again at 90°C for 20 min. Plates were cooled to room temperature on an ice block, then background absorbance was measured at 540 nm. After adding 50 μL of 0.2 mg of vanillin per 1 mL of 17% phosphoric acid solution to each well and allowing colour to develop for 10 min, final absorbance was measured at 540 nm. Samples were compared to cholesterol standards (Sigma-Aldrich; 175, 150, 125, 100, 75, 50 and 25 μg/mL). After blank subtraction, sulphuric acid background absorbance was subtracted from phosphovanillin final absorbance to calculate the concentrations.

Protein concentrations were quantified using the FluoroProfile® Protein Quantification Kit (Sigma-Aldrich). A 1:50 dilution was created from the liquid fraction of homogenized tissue. Fifty microlitres of working reagent was added to serial dilutions of the starting 1:50 dilution (100%, 25%, 6.25%, 1.5625%), and sample plates were incubated at room temperature in the dark for 30 min before measuring fluorescence at an excitation of 510 nm and an emission of 620 nm. Samples were blank subtracted and compared to 175, 150, 125, 100, 75 and 25 μg/mL bovine serum albumin standards.

Glycogen concentrations were quantified using an anthrone-based assay adapted from Leyva et al. (2008). First, we separated glycogen from total sugars in the carbohydrate fraction of homogenized tissue. Ten microlitres of 18% Na2SO4 was added to 40 μL of the liquid homogenized tissue sample, then 700 μL of 1:1 chloroform:methanol solution was added to the 50 μL solution and samples were centrifuged at 12700×g for 5 min. The supernatant containing free sugars was removed, with the glycogen remaining as a precipitate. Residual chloroform was evaporated off, then 500 μL of water was added and the sample was vortexed to resuspend the glycogen. One hundred fifty microlitres of anthrone reagent (1 mg/mL of anthrone in concentrated H2SO4) was added to 50 μL of the isolated glycogen sample. Sample plates were incubated at 4°C for 10 min, then 100°C at 300 rpm on a thermomixer, then at room temperature in the dark for 20 min. Absorption values were measured at 620 nm, blank subtracted and compared to glucose standards (Rica glucose standard at 560, 480, 400, 320, 240, 160 and 80 μg/mL).

To assess whether nutrient concentrations were associated with body size in addition to diet treatment, we measured the length of the front wing marginal cell for all queens; this is highly correlated with overall body size in bumble bees (Shpigler et al., 2013). Statistical models that included this proxy for body size, natal colony and diet treatment were assessed using Akaike information criteria (AIC) to identify best-fit linear mixed effects models in R (lme4). Tukey’s HSD post hoc analysis was used to test for significant differences between treatments. All statistical analyses in the study were performed in R (R Core Team, 2014).

Weight gain
A subset of queens was weighed repeatedly during the pre-diapause period to track changes in mass as an indirect proxy for nutrient sequestration. To examine weight change during the period (‘incremental weight gain’), a set of queens (n = 33 queens from 6 source colonies; n = 4–9 per diet treatment group) were weighed at the start (age 0 day) and end (age 11 days) of the period, and every 2–3 days in between. The number of days between weighing events was randomized with respect to treatment. Weights were then compared over time across diet treatment groups using the linear mixed effects (nlme) model with colony as a random factor. To examine total change in queen weight across the entire nutrient sequestration period (‘total weight gain’), data from these queens were combined with additional starting and ending weight data from a subset of queens also used in the survival analysis (n = 45), for a total set of 78 queens used for the total weight gain analysis. Here, the effects of treatment and natal colony on total weight gain were explored using an analysis of variance (ANOVA), with post hoc examinations of diet treatment effects using Tukey’s test. Body sizes were not recorded for this subset of bees, and thus, this factor was not included in these analyses. All weight data were normally distributed.

Survival
Queens in the survival analysis (n = 136 queens from 27 colonies; 22–37 per diet treatment group; Supplementary Table 1) were marked with individual number tags made for bee marking (purchased from Bienen-Voigt & Warnholz, Ellerau, Germany). Queens were not anesthetized or chilled for this process, and tags were attached with glue provided. At ages 5–8 days (during the feeding treatment period), they were placed in mating chambers in groups of 9–12 with 50 males for a period of 3 h daily. The cages were placed in natural light near windows. Methods for mating queens followed Röseler (1985) with the exception of the size of the mating cage (here, the chambers were approximately 1.5 ft³ with mesh sides). All queens used in this analysis were observed copulating on one or more days. Males originated from a separate set of colonies also supplied by Koppert Biological Systems. The number of times a queen mated did not influence the likelihood of surviving until the end of the experiment, irrespective of diet [generalized linear model (GLM) days mated (NA, 1, 2, 3, 4); t-values = −0.611, −1.049, −0.418, −0.204, 0.00; df = 135; Pr (>|t|) = 0.542, 0.293, 0.677, 0.838, 1.000]. At age 11 days, all live queens were placed on a bed of moistened sphagnum moss in small plastic boxes (approximately 2 in³) and stored at 4°C in total darkness for a 2-month period to simulate overwintering (Beckman et al., 1998). Humidity was not controlled, but queens were inspected weekly and their sphagnum moss was re-moistened if dry. At the end of this 2-month period, queens were inspected for mortality. Queens that survived the overwintering period (n = 59) were placed in individual wooden nest boxes (approximately 3/4 ft³) under
queen rearing conditions described above. During this 2-week post-diapause period, all queens received control diet pollen (Brushy Mountain Bee Farm, Moravian Falls, NC) and nectar _ad libitum_ and were inspected daily for mortality.

Statistical models that assessed the effects of queen diet treatment, natal colony and weight upon entering diapause on survival were compared using Akaike information criteria (AICc) to identify best-fit models using the program MuMIn. Likelihood of queen mortality was compared across the three life stages using Cox Proportional Hazards (coxph), and queen survival was also compared across the three life stages using binomial generalized linear models (GLMs). All results reported employ the use of these two best-fit models for these analyses.

**Results**

**Diet effects on nutritional status**

Diet treatment had a significant effect on relative concentrations of all three nutrients evaluated [lipid, protein and glycogen; χ²(4) = 90.467, 22.336, 53.595; P < 0.0001, 0.001, 0.0001]. The best-fit models for relative concentrations of all three nutrient classes included body size (inferred from wing marginal cell) and natal colony as random factors; however, there was not a significant effect of either of these factors on relative nutrient concentrations when the models were employed. Pollen-starved queens had relatively lower lipid (Tukey’s HSD: P < 0.0001) but not protein or glycogen concentrations (Tukey’s HSD: P = 0.07 and 0.66, respectively) than the control diet treatment group (Fig. 1A–C). Nectar-starved (0% w/v sucrose concentration) queens had significantly lower lipid and glycogen (Tukey’s HSD for both comparisons: P < 0.001), but not protein (Tukey’s HSD: P = 1.0), concentrations than the control diet treatment group (Fig. 1A–C). The low–sucrose concentration diet did not significantly impact relative glycogen levels (Tukey’s HSD: P = 0.22). Glycogen concentrations were higher in queens fed the high concentration nectar diet relative to the control group (Fig. 1C; Tukey’s HSD: P < 0.001). Relative protein concentrations were lower than the control group in the low-concentration sucrose and high–sucrose concentration queens (Tukey’s HSD: P = 0.001 and 0.002, respectively) and were particularly variable in the control diet treatment group (> 8-fold different in some samples).

**Diet effects on weight gain**

In all diet treatment groups, the greatest increase in queen weight occurred between ages 0 and 2–3 days (Tukey’s HSD: P < 0.001). Thereafter, whereas high-concentration sucrose and control diet bees maintained or increased weight beyond days 5–6, queens deprived of pollen or given low or no sucrose concentration nectar did not continue gaining weight. Nectar-starved queen weights were the most strongly impacted by diet and were significantly lower than control diet bees from...
days 5–6 through day 11 ($P < 0.005$). Queens fed the low-sucrose concentration diet had significantly lower weights than control bees at the last two time points (days 8–9 and day 11, $P < 0.05$), and pollen-starved bees were significantly lower weight than were control bees only at day 11 ($P < 0.05$) (Fig. 2A).

For the total weight gain analysis, across the entire pre-diapause period, only nectar-starved queens failed to gain substantial weight ($t = -4.152$, Pr ($>|t|) < 0.001$, Fig. 2B). Queens in all other diet treatment groups gained on average 0.15 g (approximately 25% of their average starting weight) across the 12-day pre-diapause period, versus mean weight gain of 0.05 g in the nectar-starved group. Queens fed a high-concentration (75%) nectar diet did not gain more weight across the pre-diapause period than did queens fed the control diet ($t = 0.033$, Pr ($>|t|) = 1.0$). Natal colony did not influence total weight gain ($F = 1.460, 18, 55$, Pr ($> F) = 0.142$).

**Survival across life stages**

Diet during the nutrient sequestration period (ages 0–11 days) had a strong influence on queen survival across the experiment (Fig. 3, Supplementary Table 1). During the pre-diapause (i.e. nutrient sequestration) period, during which the diet treatments were administered, no diets significantly impacted survival. Queens in the control group were 50% more likely to survive than were queens in the pollen-starved group during this period, although this difference was not statistically significant ($z$-value = 1.900, Pr ($>|z|) = 0.057$). The sugar concentration of nectar and the pollen that queens received during the nutrient sequestration period impacted survival during the subsequent life stages. Both nectar and pollen starvation significantly lowered queen survival during the diapause period ($P < 0.01$ and 0.05, respectively), whereas only the low-sugar concentration nectar diet treatment impacted queen survival during the post-diapause period ($P < 0.05$). The control and high-sugar concentration groups had overwhelmingly higher queen survival rates through the post-diapause period (> 75% survival in both groups). Natal colony (in addition to diet treatment, and the interaction between these factors) was a factor in the best-fit model in the Cox proportional hazards analysis, with some natal colonies exhibiting significantly different likelihoods of survival across the experiment. In the GLM analysis, the best-fit model included diet treatment and also queen body mass at the end of the nutrient sequestration period, the latter of which was positively associated with the likelihood of surviving to the end of the experiment ($t = -4.259$, Pr ($>|t|) < 0.001$).

**Discussion**

We experimentally manipulated the availability of pollen and the nectar diets of young adult queen bumble bees during the pre-diapause nutrient sequestration period to evaluate impacts on three nutritional phenomena: (i) nutritional state (lipids, glycogen and protein), (ii) the timescale upon which sequestration occurs and (iii) the consequences of early-life nutrition, specifically survival across the nutrient sequestration period and the subsequent diapause and post-diapause periods. Queens deprived of pollen and nectar-derived sugars exhibited relatively lower lipid amounts at the end of the pre-diapause period, whereas queens deprived of nectar-derived sugars had lower glycogen amounts. Queens fed the highest (75%) nectar sucrose concentration had higher glycogen stores than the control group (fed a 50% sucrose solution), suggesting that queens can gain additional nutrient storage benefits when they have access to nectar at the highest end of the naturally occurring spectrum of nectar.
Sugar concentrations. This concentration is similar to that of stored nectar found in honey pots in nests (reported as 70–87% sugar; Knee and Medler, 1965; Crane 1972), which is what queens most likely feed on during the first few days of adulthood in their natal nests. With respect to pollen, the total amount that a queen may need to consume for optimal fat and protein intake is likely to be highly dependent on the floral resources her pollen diet is derived from, given that the relative abundance of these nutrients in pollen can vary widely across, and even within, flowering plant species (Roulston and Cane, 2000). The methodology we used to quantify nutrient amounts allowed us to identify whether queens were relatively deficient in particular macronutrients as a function of the diet they received before entering diapause. Future studies might also examine nutrition more holistically, to identify absolute threshold levels of stored nutrients that are necessary for life stage survival, and the relationships between macronutrient classes and their storage and metabolism.

Queens in our study gained the most weight between the ages of 0 and 2–3 days. This indicates that these first few days of adulthood are potentially the most important for nutrient sequestration in bumble bee queens. This finding is consistent with earlier work by Alford (1969b) and Röseler and Röseler (1986), who also found that pre-diapause increases in stored lipids and glycogen are most dramatic in the first few days of adult life. The narrow timing of this critical nutrient sequestration period, and our finding that glycogen sequestration can be extremely limited when queens do not have access to sufficient dietary carbohydrates, has implications related to the nutritional ecology of wild bumble bees. Queens typically spend the first few days of adulthood in their natal nests, presumably undergoing maturational processes in preparation for flight and the other activities (including mating) they engage in upon leaving the nest (Alford, 1969b; Alford, 1975; Röseler and Röseler, 1986). As such, the pollen and nectar resources from which queens obtain the majority of nutrients for pre-diapause sequestration are collected by their sister-workers, who forage in the area surrounding the natal colony. This suggests that floral resources within the foraging range of worker bumble bees (estimated to be at a radius of up to 9–10 km around nests; reviewed in Woodard et al., 2015) are relevant to the long-term survival of queens, in particular nectar resources available prior to or during late summer or early fall, when new queens begin emerging from nests. Conservation strategies focused on pollinator foraging habitat might incorporate late-blooming forage into hedgerows, seed mixes and other pollinator habitat enhancements to improve food availability for bumble bee queens during this important period. Bumble bees store food resources in the nest, but typically only enough to feed the colony for a few days (Heinrich 2004; Cartar and Dill, 1990); this might further limit food resources available to newly eclosed queens in habitats with fewer or lower-quality resources. It is unknown how flexible the period of nutrient sequestration is, for example, whether queens can compensate for insufficient food resources during their first few days of adult life by sequestering nutrients later during the pre-diapause period. Queens have also been reported to fill their crops (or ‘honey stomachs’: a specialized foregut segment) with nectar prior to overwintering, up to almost a third of their body weight (Alford 1969a). This represents an alternative nutrient storage mechanism that may serve in part to offset failure to sequester sufficient nutrients in the fat body or muscle tissue.

We also found evidence that in addition to immediate effects on nutrient sequestration (i.e. during the pre-diapause period), diet also has both immediate and persistent effects on queen survival. Availability of pollen during the pre-diapause period may be more important for surviving this
period, although this finding was not statistically significant ($P = 0.057$). Adult worker bumble bees consume pollen (Vaudo et al., 2016a, 2016b) and exhibit lower survivorship under pollen starvation conditions (Smeets and Duchateau, 2003), but pollen-feeding in young, non-reproductive queens has been thus far relatively unexplored. Given that approximately half of queens (52%) in the pollen-starved group were able to survive the pre-diapause period, there may be individual differences in the nutritional needs of young queens, perhaps driven by differences in diet and nutrition during the larval stage. This mortality might also have been driven by an interaction between diet and other factors that are harmful to bumble bees, such as pathogens (Brunner et al., 2014; Conroy et al., 2016; Di Pasquale et al., 2013). During the diapause period, both pollen and nectar limitation had particularly strong effects on survival, and thus although the primary nutrients sequestered from these food resources differ (lipids and protein vs. carbohydrates, respectively), their unavailability may have similar consequences for queen fitness during this life stage. Finally, during the post-diapause period, it was nectar sugar concentration that impacted queen survival, despite all queens in our experiment being provided with the control diet during the post-diapause period. Thus, there may be holdover effects of poor pre-diapause nutrition after the diapause period, even if queens have access to improved food resources after diapause. Spring queens resume feeding upon emergence from diapause and require nectar-derived carbohydrates to sustain extensive flight activity for dispersal and to fuel nest-provisioning activities (Heinrich, 2004), and also pollen and nectar to develop their ovaries (Vogt et al., 1998). A spring queen’s ability to successfully initiate a nest and produce offspring is further strained by stressors such as parasites (Rutrecht and Brown, 2008) and neonicotinoid pesticides (Baron et al., 2017; Leza et al., 2018). Thus, it appears that bumble bee queens need access to high-quality pollen and nectar resources before entering diapause, residual nutrient stores when they emerge from diapause and also access to high-quality food resources after emerging from diapause, in order to survive and successfully establish nests under ecologically realistic conditions.

Queens in our experiment were maintained under relatively low energy expenditure conditions, with food provided ad libitum in their nesting cages. The impacts of diet on nutrient sequestration and survival are likely much stronger in wild queens, who expend more energy during dispersal before and after diapause and foraging during nest-founding, and therefore have greater energetic demands. Wild queens also undergo a much longer overwintering period than in our experiment, of up to 6–9 months (Alford, 1969a; Szabo and Pengelly, 1973), versus 2 months in our experiment. They also experience fluctuating temperature conditions in the wild during the diapause period, which may influence the rate at which stored nutrients are metabolized (Vesterlund and Sorvari, 2014; Vesterlund et al., 2014). In the wild, queens may also be able to access alternative food sources to compensate for poor-quality diets, for example by seeking out higher-quality foraging habitats. Because queens can disperse more than 9 km (Lepais et al., 2010), they may be able to access food resources across considerably larger spatial scales once they depart their natal nests. Recent work on bumble bee workers suggests that they are able to access the quality of food resources and use this information to make feeding- or foraging-related decisions (Francis et al., 2016; Muth et al., 2016; Ruedenauer et al., 2015; Ruedenauer et al., 2016; Vaudo et al., 2016a). Queens likely have similar sensory abilities and the potential to make decisions that improve their nutrient intake. Mortality during diapause was fairly high in our study, even among queens given the control and high concentration nectar diets (e.g. 59% and 61% diapause survival, respectively). Previous laboratory studies have seen much higher (> 80%) diapause survival in B. terrestris, using similar overwintering durations and temperatures (Beekman et al. 1998; Gosterit and Gurel, 2009). How these laboratory survival rates compare to overwintering mortality in wild queens, and how this differs between species, is not currently known.

Overall, our results suggest that diet composition significantly influences nutrient sequestration and survival in queen bumble bees, with broader implications for wild bumble bee conservation efforts. Whereas the overwhelming majority of bumble bee research has focused on the worker caste, there may be special nutritional considerations for queen bumble bees related to caste-specific aspects of their nutritional physiology, such as their caste-specific need to sequester nutrients prior to entry into diapause. The pre-diapause nutrient sequestration period, and in particular the first few days of adulthood, may be a critical window of opportunity during queen development where food resources are particularly important, and insufficient food resources may have especially profound impacts on bumble bee populations. To counteract global declines in bumble bees (Goulson et al., 2015; 2008a; Williams and Osborne, 2009), strategies for managing bee foraging habitat should ideally consider nutritional needs (Vaudo et al., 2015; Woodard and Jha, 2017). Our findings suggest that consistent access to pollen and nectar resources late in the nesting season, as well as during early spring, may be important for promoting queen bumble bee survival. Thus, the results of our study have critical implications for the management of floral food resources for bumble bees, in light of the unique nutritional biology of the queen caste.

Supplementary material
Supplementary material is available at Conservation Physiology online.

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References
Alford DV (1969a) A study of the hibernation of bumblebees (Hymenoptera: Bombidae) in southern England. J. Animal Ecol. 38: 149–170.
Alford DV (1969b) Studies on the fat-body of adult bumble bees. J. Api. Res. 8: 37–48.
Alford DV (1975) Bumble Bees. Davis-Poynter Limited, London, UK
Baker HG, Baker I (1983) A brief historical review of the chemistry of floral nectar. In B Bentley, T Elias, eds, The Biology of Nectaries. Columbia University Press, New York, pp. 126–152
Baron GL, Raine NE, Brown MJF (2017) General and species-specific impacts of a neonicotinoid insecticide on the ovary development and feeding of wild bumblebee queens. Proc. Roy. Soc. Lond. B: Biol. Sci. 284: 20170123–20170128.
Beekman M, Stratum P, Lingeman R (1998) Diapause survival and post-diapause performance in bumblebee queens (Bombus terrestris). Entomol. Exp. et App. 89: 207–214.
Brunner FS, Schmid-Hempel P, Barribaleau SM (2014) Protein-poor diet reduces host-specific immune gene expression in Bombus terrestris. Proc. Roy. Soc. Lond. B: Biol. Sci. 281.
Burkle LA, Marlin JC, Knight TM (2013) Plant-pollinator interactions over 120 years: loss of species, co-occurrence, and function. Science 339: 1611–1615.
Cartar RV, Dill LM (1990) Colony energy requirements affect the foraging currency of bumble bees. Behav. Ecol. Sociobiol. 27: 377–383.
Chalcoff VR, Aizen MA, Galetto L (2006) Nectar concentration and composition of 26 species from the temperate forest of South America. Annals of Botany 97: 413–421.
Cheng Y-S, Zheng Y, VanderGheynst JS (2011) Rapid quantitative analysis of lipids using a colorimetric method in a microplate format. Lipids 46: 95–103.
Conroy TJ, Palmer-Young EC, Irwin RE, Adler LS (2016) Food limitation affects parasite load and survival of Bombus impatiens (Hymenoptera: Apidae) infected with Cribidia (Trypanosomatida: Trypanosomatidae). Environ. Entomol. 45: 1212–1219.
Corbet SA, Williams IH, Osborne JL (1991) Bees and the pollination of crops and wildflowers in the European Community. Bee World 72: 47–49.
Crane E (1972) Bee products. Bee World 53: 38–39.
Daugherty THF, Toth AL, Robinson GE (2011) Nutrition and division of labor: effects on foraging and brain gene expression in the paper wasp Polistes metricus. Mol. Ecol. 20: 5337–5347.
De Luca PA, Vallejo-Marín M (2013) What’s the ‘buzz’ about? The ecology and evolutionary significance of buzz-pollination. Curr. Opin. Plant Biol. 16: 429–435.
Di Pasquale G, Salignon M, Le Conte Y, Belzunces LP, Decourtey A, Kretzschmar A, Suchail S, Brunet J-C, Alaux C (2013) Influence of pollen nutrition on honey bee health: do pollen quality and diversity matter? PLoS ONE 8: e72016.
Fliszkiewicz M, Wilkaniiec Z (2007) Fatty acids and amino acids in the fat body of bumblebee Bombus terrestris (L) in diapausing and non-diapausing queens. J. Api. Sci. 51: 59–63.
Francis JS, Muth F, Papaj DR, Leonard AS (2016) Nutritional complexity and the structure of bee foraging bouts. Behav. Ecol. 27: 903–911.
Gosterit A, Gurel F (2009) Effect of different diapause regimes on survival and colony development in the bumble bee, Bombus terrestris. J. Apicult. Res. 48: 279–283.
Goulson D (2010) Bumblebees: Behaviour, Ecology, and Conservation, EdSecond. Oxford University Press, Oxford
Goulson D, Lye GC, Darvill B (2008a) Decline and conservation of bumble bees. Annu. Rev. Entomol. 53: 191–208.
Goulson D, Lye GC, Darvill B (2008b) Diet breadth, coexistence and rarity in bumblebees. Biodivers. Conserv. 17: 3269–3288.
Goulson D, Nicholls E, Botias C, Rotheray EL (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. Science 347: 1255957–1255957.
Heinrich B (2004) Bumblebee Economics, Ed2nd. Harvard University Press, Cambridge
Holm SN (1972) Weight and life length of hibernating bumble bee queens (Hymenoptera: Bombidae) under controlled conditions. Insect Syst. & Evol. 3: 313–320.
Knee WJ, Medler JT (1965) Sugar concentration of bumble bee honey. Am. Bee J. 105: 174–175.
Kremen C, Bugg RL, Nicola N, Smith SA, Thorp RW (2002) Native bees, native plants and crop pollination in California. Fremontia 30: 41–49.
Lepais O, Darvill B, O’Connor S, Osborne JL, Sanderson RA, Cussans J, Goffe L, Goulson D (2010) Estimation of bumblebee queen dispersal distances using sibship reconstruction method. Mol. Ecol. 19: 819–831.
Leyva A, Quintana A, Sánchez M, Rodríguez EN, Cremata J, Sánchez JC (2008) Rapid and sensitive anthrone-sulfuric acid assay in microplate format to quantify carbohydrate in biopharmaceutical products: method development and validation. Biologicals 36: 134–141.
Leza M, Watrous KM, Bratu J, Woodard SH (2018) Effects of neonicotinoid insecticide exposure and monofloral diet on nest-founding bumblebee queens. *Proc. Roy. Soc. Lond. B: Biol. Sci.* 285: 20180761.

McMahon A, Lu H, Butovich IA (2013) The spectrophotometric sulfophospho-vanillin assessment of total lipids in human meibomian gland secretions. *Lipids* 48: 513–525.

Memmott J, Waser NM, Price MV (2004) Tolerance of pollination networks to species extinctions. *Proc. Roy. Soc. Lond. B: Biol. Sci.* 271: 2605–2611.

Muth F, Francis JS, Leonard AS (2016) Bees use the taste of pollen to determine which flowers to visit. *Biol. Letters* 12: 20160336.

Piidal A, Hofbauer J (1996) Laboratory rearing and nutrition of young queens of bumblebee (*Bombus terrestris* (L)) from emergence to diapause. *Sci. Studies Res. Inst. Fodder Plants Troubsko* 14: 125–130.

R Core Team (2014) *R*: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna.

Röseler PF, Röseler I (1986) Caste specific differences in fat body glycerol metabolism of the bumblebee. *Bombus terrestris*. *Insect Biochem.* 16: 501–508.

Roulston TH, Cane JH (2000) Pollen nutritional content and digestibility for animals. *Plant Syst. Evol.* 222: 187–209.

Ruedenauer FA, Spaethe J, Leonhardt SD (2015) How to know which food is good for you: bumblebees use taste to discriminate between different concentrations of food differing in nutrient content. *J. Exp. Biol.* 218: 2233–2240.

Ruedenauer FA, Spaethe J, Leonhardt SD (2016) Hungry for quality—individual bumblebees forage flexibly to collect high-quality pollen. *Behav. Ecol. Sociobiol*. 70: 1209–1217.

Rutrecht ST, Brown MJF (2008) The life-history impact and implications of multiple parasites for queen bumble bees. *Int. J. Parasitol.* 38: 799–808.

Shpigler H, Tamarkin M, Gruber Y, Poleg M, Seigal AJ, Bloch G (2013) Social influences on body size and developmental time in the bumblebee *Bombus terrestris*. *Behav. Ecol. Sociobiol.* 67: 1601–1612.

Sladen FWL (1912) *The Humble-Bee: Its Life History and How to Domesticate It*. Macmillan, London.

Smeets P, Duchateau KM (2003) Longevity of *Bombus terrestris* workers (Hymenoptera: Apidae) in relation to pollen availability, in the absence of foraging. *Apidologie* 34: 333–337.

Stubbcs CS, Drummond FA (2001) *Bombus impatiens* (Hymenoptera: Apidae): an alternative to *Apis mellifera* (Hymenoptera: Apidae) for low-bush blueberry pollination. *J. Econ. Entomol.* 94: 609–616.

Suzuki Y, Kawaguchi LG, Munidasa DT (2009) Do bumble bee queens choose nest sites to maximize foraging rate? Testing models of nest site selection. *Behav. Ecol. Sociobiol.* 63: 1353–1362.

Svensson B, Lagerlof J, Svensson BG (2000) Habitat preferences of nest-seeking bumble bees (Hymenoptera: Apidae) in an agricultural landscape. *Agric. Ecosys. Environ.* 77: 247–255.

Szabo TI, Pengelly DH (1973) The over-wintering and emergence of *Bombus (Pyrobombus) impatiens* (Cresson) (Hymenoptera: Apidae) in Southern Ontario. *Insect. Soc.* 20: 125–132.

Toth AL, Kantarovich S, Meisel AF, Robinson GE (2005) Nutritional status influences socially regulated foraging ontogeny in honey bees. *J. Exp. Biol.* 208: 4641–4649.

Vaudo AD, Patch HM, Mortensen DA, Tooker JF, Grozinger CM (2016a) Macronutrient ratios in pollen shape bumble bee (*Bombus impatiens*) foraging strategies and floral preferences. *Proc. Natl. Acad. Sci. U.S.A.* 113: E4035–E4042.

Vaudo AD, Stabler D, Patch HM, Tooker JF, Grozinger CM, Wright GA (2016b) Bumble bees regulate their intake of the essential protein and lipid pollen macronutrients. *J. Exp. Biol.* 219: 3962–3970.

Vestergaard S-R, Sorvari J (2014) Longevity of starved bumblebee queens (Hymenoptera: Apidae) is shorter at high than low temperatures. *Eur. J. Entomol.* 111: 1–5.

Vestergaard S-R, Lilley TM, van Ooik T, Sorvari J (2014) The effect of overwintering temperature on the body energy reserves and phenoloxidase activity of bumblebee *Bombus lucorum* queens. *Insect. Soc.* 61: 265–272.

Vogt FD, Heinrich B, Plowright C (1998) Ovary development in bumble bee queens: the influence of abdominal temperature and food availability. *Can. J. Zool.* 76: 2026–2030.

Votavová A, Tomčalová A, Kofroňová E, Kudzejová M, Šobotník J, Jiří P, Komzákova O, Valterová I (2015) Seasonal dynamics in the chemistry and structure of the fat bodies of bumblebee queens. *PloS one* 10: e0142261.

Watrous KM, Duennes MA, Woodard SH (2019) Pollen diet composition impacts early nesting success in queen bumble bees *Bombus impatiens* Cresson (Hymenoptera: Apidae). *Environ. Entomol*. nVZ043.

Williams PH, Osborne JL (2009) Bumblebee vulnerability and conservation world-wide. *Apidologie* 40: 367–387.

Woodard SH (2017) Bumble bee ecophysiology: integrating the changing environment and the organism. *Curr. Opin. Insect Sci.* 20: 101–108.

Woodard SH, Jha S (2017) Wild bee nutritional ecology: predicting pollinator population dynamics, movement, and services from floral resources. *Curr. Opin. Insect Sci.* 23: 83–90.

Woodard SH, Lozier JD, Goulson D, Williams PH, Strange JP, Jha S (2015) Molecular tools and bumble bees: revealing hidden details of ecology and evolution in a model system. *Mol. Ecol.* 24: 2916–2936.