Journal of Apicultural Research

ISSN: 0021-8839 (Print) 2078-6913 (Online) Journal homepage: https://www.tandfonline.com/loi/tjar20

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To cite this article: Susma Giri, Basant Giri & Michael E. Dillon (2019) An optimized approach for extraction and quantification of energy reserves in differentially fed bumble bees (*Bombus*), Journal of Apicultural Research, 58:4, 531-541, DOI: 10.1080/00218839.2019.1614728

To link to this article: https://doi.org/10.1080/00218839.2019.1614728

Published online: 30 May 2019.

Journal of Apicultural Research, 2019
Vol. 58, No. 4, 531–541, https://doi.org/10.1080/00218839.2019.1614728

ORIGINAL RESEARCH ARTICLE

An optimized approach for extraction and quantification of energy reserves in differentially fed bumble bees (*Bombus*)

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(Received 4 December 2017; accepted 19 March 2019)

Recent declines in managed and native bee populations are likely driven by diverse causes, including pesticides, parasites, habitat loss, and changing climate. The effects of these drivers may be all mediated, in part, via effects on energy reserves which are primarily lipids, sugars, and glycogen. Changes in energy reserves can be a useful indicator of individual and population-level bee health. Here we describe an approach for estimating quantities of lipids, sugars, and glycogen from differentially fed individuals of bumble bees (genus *Bombus*). For both lab-reared (*Bombus impatiens*) and field-collected (*Bombus huntii*) bumble bees, this approach reliably detected small shifts in energy reserves. The inexpensive and straightforward approach described here should be more generally useful for monitoring health of bee populations and other insect species.

Keywords: bee nutrition; bee health; lipids; carbohydrates; colorimetric assay

Introduction

Recent declines in managed bee colonies (Neumann & Carreck, 2010; van Engelsdorp, Hayes, Underwood, & Pettis, 2008) and in wild bee populations (Biesmeijer et al., 2006; Cameron et al., 2011) have engendered concern about the future of these critical pollinators. Declines in bee populations may drive parallel declines in the plants that depend on them, with serious ecological and economic implications (Biesmeijer et al., 2006; Gallai, Salles, Settele, & Vaissière, 2009; Losey & Vaughan, 2006).

Bee population declines are likely driven by diverse and interacting factors, including habitat loss (Winfree, Aguilar, Vázquez, LeBuhn, & Aizen, 2009), pesticide use (Gill, Ramos-Rodriguez, & Raine, 2012; Whitehorn, O'Connor, Wackers, & Goulson, 2012), resource availability (Cane & Tepedino, 2017), pathogens (e.g., Goulson, Lye, & Darvill, 2008; Higes et al., 2008), and changing climate (Potts et al., 2010; reviewed in P. H. Williams & Osborne, 2009). These factors drive bee declines, in part, due to their direct and indirect impacts on physiology (Giannini et al., 2012; Hegland, Nielsen, Lázaro, Bjerknes, & Totland, 2009), particularly through effects on available and stored energy (lipids and carbohydrates). This energy is critical for, among other things, regulating individual and colony temperatures, defending the colony from intruders (Cartar & Dill, 1991), incubating brood (Heinrich, 1975), resisting pathogen attack (Huang, 2012), and maintaining immunity (Alaux, Ducloz, Crauser, & Conte, 2010). Bees obtain these resources primarily from the flowers. Flower nectar serves as the main source of carbohydrates and pollen as the source for lipids, proteins and other micronutrients in bees (Nicolson & Thornburg, 2007; Roulston & Cane, 2000; Vaudo, Tooker, Grozinger, & Patch, 2015). As such, landscape level changes in floral resources, brought about by land-use and climatic changes (e.g., Biesmeijer et al., 2006; Carvell et al., 2006; Potts et al., 2010), among others, combined with concomitant increases in the rate of resource use brought about by an increased metabolism (Dillon, Wang, & Huey, 2010) may challenge energy balance, reducing bee vigor through shortfalls in energy reserves. The diverse drivers of bee population declines are all likely mediated, in part, by effects on stored energy (e.g., Czerwinski & Sadd, 2017); measurement of nutritional status is therefore an important tool for diagnosing and studying bee declines.

Nutritional status is indicated, to a certain degree, by the relative quantities of lipids and carbohydrates (sugars and glycogen), which are critical metabolic substrates in insects (Arrese & Soulages, 2010; Marron, Markow, Kain, & Gibbs, 2003; Ohtsu, Kimura, & Hori, 1992). Whereas lipids provide efficient energy storage, help prevent cuticular water loss, and are integral to the functioning of cell membranes (Downer & Matthews, 1976), sugars and glycogen fuel metabolism and are necessary for the synthesis of cryoprotectants (J. M. Storey & Storey, 1986; K. B. Storey, 1997). Quantification of lipids, sugars, and glycogen therefore provides a useful tool for monitoring insect health (e.g., Olson, Fadamiro, Lundgren, Natha, & Heimpel,

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2000; Lee, Heimpel, & Leibee, 2004; Lee & Heimpel, 2008).

Accurate estimation of the energy components depends partly on effective extraction of these compounds from tissues. Whereas there are various methods for extraction of the energy components (e.g., petroleum-ether method, O'Neill, O'Neill, Kemp, & Delphia, 2011), here we focus on a commonly used extraction procedure for insects that was first developed for studies of mosquitoes (Van Handel, 1965; Van Handel & Day, 1988) and has since been used for diverse insects (e.g., Ahsaei, Tabadkani, Hosseininaveh, Allahyari, & Bigham, 2013; Fotouhi, Fazel, & Kavousi, 2015; Zhang, Liu, Wang, Wan, & Li, 2011). Briefly, insect tissue is homogenized in sodium sulfate (Na_2SO_4) with a few drops of methanol and then placed in a chloroform:methanol (1:1) solution; lipids and sugars dissolve in the polar layer and the sodium sulfate together with methanol causes glycogen to precipitate out of solution. After centrifugation, the polar layer with lipids and sugars and precipitated glycogen pellet can be subsequently quantified. However, in initial experiments with bees, excessive tissue residue collected in the pellet, precluding subsequent glycogen quantification reactions.

Once extracted, energy components can be quantified using a number of methods (reviewed by Chaplin, 2006; C. M. Williams, Thomas, MacMillan, Marshall, & Sinclair, 2011). Colorimetric assays are commonly used to quantify energy components because of their simplicity, convenience, low cost, high throughput and most importantly, reasonable accuracy and precision (Foray et al., 2012). The sulpho-phospho vanillin (SPV) assay for quantification of lipids (Knight, Anderson, & Rawle, 1972) is rapid, reliable, and requires minimal sample preparation (Cheng, Zheng, & VanderGheynst, 2011). Similarly, the anthrone and phenol methods are reliable and straightforward approaches to quantifying carbohydrates (Laurentin & Edwards, 2003; Masuko et al., 2005).

A well-established method for extraction and quantification of energy reserves in insects as small as mosquito has been described by Van Handel (1965) and Van Handel & Day (1988). A few modifications of the extraction and quantification approach following these established methods have been described. The most recent publications of interest are by Cheng et al. (2011) and Foray et al. (2012). Both these methods provide the advantage of small volume and high throughput. Further, method by Foray et al. (2012) also provides the benefit of extracting and quantifying proteins in addition to lipids and carbohydrates in the same individual smaller than 15 mg (but can be used for larger individuals with modifications). However, both these methods have their own disadvantages. The use of organic solvents in method by Cheng et al. (2011) appear to corrode the polystyrene microplate wells leading to inconsistencies and pronounced variation in optical density (OD) readings. Whereas this problem has been solved by Foray et al. (2012) with the use of borosilicate microplates for the quantification of carbohydrates, performing this method requires relatively more resources and technical knowledge compared to the one we propose in this paper.

Here, we report the optimization for single bumble bee individuals of a previously used approach for the extraction and quantification of the three major energy components (modified from Van Handel, 1965; lipids, sugars, and glycogen; Van Handel & Day, 1988) using a microplate reader (an instrument that provides photometric measurements of multiple samples in contrast to traditional spectrophotometer that provides reading for one sample at a time). We first evaluate different approaches for quantification of lipids and carbohydrates in bees and then validate the methods by estimating the quantities of lipids, sugars and glycogen in both lab-reared and field-collected bumble bees (*Bombus impatiens* and *Bombus huntii*, respectively, 55 mg–250 mg dead weight but can be applied to smaller or larger bees by either concentrating or by diluting the extracts) differing in nutritional status.

Our method provides the advantage of being relatively simple with good throughput, which can be performed by anyone with a moderate knowledge in lab experiments. With a simple and appropriate training, anyone can easily operate the microplate reader. Therefore, in addition to the researchers, this method may also be useful to the general bee biologists who would want to gain better idea of bee nutrition physiology.

Materials and methods

Extraction and separation of lipids and carbohydrates

To optimize extraction procedures for bees, we used *B. impatiens* from commercial lab-reared hives (Biobest, International Technology Services, Leamington, ON, Canada), euthanized in cyanide and stored at -20 °C until analysis. We modified the approach of Van Handel (1965) and Van Handel & Day (1988) as compiled by Benedict (2007) to extract lipids and carbohydrates. Basically, we first lyophilized (freeze-dried; Freezone 4.5, Labconco, Kansas City, USA) the dead *B. impatiens*. In contrast to the heat-drying method, lyophilization, as a result of cold-temperature, helps retain the chemical properties of the sample. We then homogenized the sample using a glass rod in a 15-mL conical glass centrifuge tube. After adding 0.2 mL of 2% Na_2SO_4 solution and 2.8 mL of 1:1 chloroform:methanol and vortexing for 3–5 s, we centrifuged the mixture for 1 min at 3000 rpm (Thermo Scientific Sorvall Legend XT/XF). This step led to the separation of tissue (including pieces of cuticle) and glycogen in the precipitate pellet from lipids and sugars in solution. The supernatant (containing lipids and sugars) was then transferred to clean tubes. We added 1 mL deionized (DI) water to the tube containing tissue and glycogen precipitate, vortexed, and then heated at 90 °C for 4 min to dissolve the glycogen (see Panzenböck and Crailsheim 1997). The solution was then centrifuged (1 min at 3000 rpm), and the supernatant (with dissolved glycogen) was transferred to a clean tube for subsequent quantification (see below). This procedure was repeated three times for each sample to completely extract glycogen from bits of tissue and cuticle (in preliminary tests, very little glycogen remained after the third extraction). To the tube containing lipids and simple sugars, we added 2 mL DI water and vortexed and centrifuged (1 min at 3000 rpm) to separate the solution into a top layer containing sugars (methanol + water + sugars) and a bottom layer containing lipids (chloroform + lipids). The three extracts (lipids, sugars, and glycogen) were then either analyzed immediately following extraction, or stored at –20 °C until analysis (usually within a week). We recorded the volumes of the layers prior to quantification of lipids and sugars to allow for subsequent calculations of whole body energy stores because only a fraction of the entire volume was used for quantification.

Quantification of energy components

Lipids

We employed the SPV assay to estimate lipid quantity. Briefly, concentrated sulfuric acid reacts with carbon-carbon double bonds in lipids to form carbonium ions. In parallel, phosphoric acid reacts with the hydroxyl group on vanillin to form a phosphate ester, increasing the reactivity of the vanillin carbonyl group. Carbonium ions react with this carbonyl group to form a colored compound (Knight et al., 1972). The OD of the colored compound is then compared to known standards to estimate lipid quantity. Standards used included soybean oil, triolein (TRO), and tripalmitin (TRP).

We evaluated two approaches to using the SPV assay to quantify lipids in bees: a high-throughput, small volume, low waste approach in which all reactions occur in a microplate well ("microplate method"; Cheng et al., 2011) and a medium throughput, high-volume SPV assay as described by Van Handel (1985a; "test-tube method") with reactions in 15 mL conical glass centrifuge tubes (Kimble Chase, NJ, USA), but with OD of reactant products measured in microplates (C. M. Williams et al., 2011). To determine the optimum wavelength (λ_{max}) for OD measurement, we first scanned wavelengths from 475 to 700 nm (2 nm increments, Tecan microplate reader, TECAN, Switzerland) for a standard soybean oil solution (1.8 mg/mL in 1:1 chloroform:methanol). We next generated standard curves using soybean oil solutions ranging in concentrations from 0.039 to 1.25 mg/mL. To test the reproducibility of the method, we ran three trials with the same soybean oil concentrations, generating three calibration curves. Standard curves were also generated for tripalmitin (TRP, 99% purity, Acros organics, saturated fatty acid "SFA"), triolein (TRO, 97% purity, Acros organics, unsaturated fatty acid) and a 50:50 mixture of tripalmitin and triolein. Each sample was measured in triplicate. A step-by-step procedure for the quantification of lipids following test-tube method is given below:

1. Place 100 µL of the lipid extract (or standard mixture) into each of three graduated centrifuge tubes with at least 5 mL capacity (triplicates).
2. Place in a heating block (block with holes to fit individual test tubes) at 90 °C to evaporate the solvent. This usually takes about 10 min.
3. Remove from the heating block, add 200 µL of concentrated sulfuric acid, and then incubate again in the heating block at 90 °C for 10 min.
4. Add vanillin reagent up to the 5-mL level and mix.
5. Remove from the heating block and allow it to cool.
6. Transfer 200 µL of the mixture into the microplate 10 min after vanillin-reagent addition.
7. Measure OD at 540 nm after 20 min of vanillin-reagent addition (it is important to keep this reaction time consistent between samples and runs).

Carbohydrates

We compared two approaches to carbohydrate quantification in bees: a phenol-sulfuric acid assay ("phenol method"; Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) carried out in microplates following Masuko et al. (2005), and a slightly modified approach to Van Handel's (1985b) anthrone-sulfuric acid assay ("anthrone method" hereafter).

The phenol method is a straightforward and reliable approach to quantifying sugars and their methyl derivatives, oligosaccharides, polysaccharides, glycolipids, proteoglycans and glycoproteins (Dubois et al. 1956; Masuko et al. 2005). When saccharides containing potential keto or aldehyde groups are heated with concentrated sulfuric acid they form furfural derivatives that condense with phenol to form colored complexes (Rao and Pattabiraman 1989) , the OD of which depends on sugar quantity. Comparison of OD measurements with known standards then allows for quantification of sugars. We determined the λ_{max} by an absorbance scan (475–750 nm in 2 nm steps) of 0.125 mg/mL glucose in DI water and generated standard curves ranging in concentrations from 0.031 to 4 mg/mL.

In the anthrone method, the strong acidic environment in combination with heat leads to hydrolysis of glycosidic bonds as well as dehydration of monomers to produce furfuraldehyde derivatives which react with anthrone to produce colored compounds (Laurentin and Edwards 2003). Comparison of OD measurements with known standards then allows for quantification of carbohydrates. As for lipids, reactions occurred in 15 mL conical glass centrifuge tubes, but OD of reactant products was measured in microplates. The optimum wavelength was obtained by a preliminary absorbance scan (475–700 nm in 2 nm steps) of a glycogen sample. We generated a standard curve for carbohydrate quantification using known glucose solutions ranging in concentration from 0.031 to 4 mg/mL. A step-by-step procedure for quantification of carbohydrates following the anthrone method is shown:

1. Place 100 μL of the carbohydrate extract (or standard mixture) into each of three graduated centrifuge tubes with at least 5 mL capacity. Add anthrone reagent up to the 5-mL level.
2. Incubate the mixture at 90 °C for 17 min (water bath is suggested although we used the heating block with holes to fit individual test tubes).
3. Immediately transfer 200 μL of the solution into wells on the microtiter plate.
4. Measure OD at 625 nm, 20 min after transferring to the microplate (keep this reaction time consistent between samples and runs).

Validation of the method by measuring energy reserves in differentially fed Bombus spp

For a number of reasons (see Results), we settled on the test-tube and anthrone methods (modified versions) for measurement of lipids and carbohydrates, respectively, in bees. To verify that this approach can detect expected, ecologically-realistic differences in energy stores, we measured total lipids, sugars, and glycogen in lab-reared and field-collected bumble bees.

First, two *B. impatiens* hives were reared in the lab. Bees had *ad libitum* access to nectar (Biogluc as provided with the Biobest hive via a supply under the nest box) and fresh pollen (replaced every 2–3 d) from 12 artificial flowers inside a foraging chamber (1 m X 0.6 m X 0.6 m) connected to the nest box by 1.59 cm polystyrene tubing. Artificial flowers were 5-dram plastic vials attached to blue plastic weigh boats (4.3 cm X 4.3 cm X 0.7 cm) via plastic tubing. To characterize a control ("fed") nutritional condition, we measured energy stores of 10 bees euthanized immediately after collection from the foraging chamber (with *ad libitum* access to the nectar). We compared these fully-fed bees with 10 "starved" bees which were collected from the foraging chamber, weighed, placed individually in small plastic vials (18.5 mL, Thornton Plastic CO., UT, USA) without food for water until they died after 43 to 48 h, and then stored at –20 °C until analysis.

We also collected thirty male *B. huntii* by visual netting in September 2013 from the University of Wyoming campus (2050 m asl; 41.3131°N 105.5814°W). We estimated the energy reserves in three treatment groups in these male bees: "field" bees that were euthanized immediately after capture, "fed" bees that were euthanized 48 h after being placed in a plastic chamber (18.7 cm × 14.3 cm × 8.3 cm, Sterilite, MA, USA) in the lab (~21 °C) provided with *ad libitum* access to 50% sugar water with additives (1% honey B healthy essence and 0.5% amino-B booster, HONEY-B-HEALTHY, Inc. Cumberland, MD, USA) and "starved" bees that died within 21 h of being placed in plastic chambers in the lab without pollen or nectar.

We measured wet weight (Acculab ALC-210.4, NY, USA, ±0.1 mg) prior to storing bees at –20 °C until measurement of energy components. We also measured dry weight after bees were lyophilized for 48 h (preliminary experiments suggested that bee tissue mass did not change after ~ 40 h), and estimated total body water as the difference between wet and dry weights. Energy components were then extracted and quantified following the optimized protocol as described above.

Statistical analyses

We used linear regressions to compare among lipid and carbohydrate standard curves to determine the appropriate method for quantification approach of each energy component. We compared wet and dry weights, and proportions (of dry weight) of energy components among treatment groups for both laboratory and field-caught bees using Welch two-sample *t*-tests and ANOVA with TukeyHSD *post hoc* comparisons, respectively. Value for water concentration is presented as percent of wet weight and values for energy components are presented as percent of dry weight. Percentages were arcsine square root transformed prior to analysis. Reported values represent the mean for triplicate measurements. All analyses and figures were done in R (R Core Team, 2016).

Results

Absorbance scans

For lipid quantification using the microplate method (Cheng et al., 2011), OD values peaked at 540 nm (Figure 1), closely matching the value reported by Cheng *et al.* (2011). We therefore used this optimal wavelength (λ_{max}) for all subsequent measurements of OD following the microplate SPV assay. For lipid quantification using the modified test-tube method (Van Handel, 1985b), OD values peaked at 534 nm (Figure 1), considerably lower than the peak wavelength of 625 nm reported by Van Handel (1985a). However, a more recent study that, similar to our approach, ran the SPV assay in glass test tubes and then subsequently transferred solutions to microplates for OD measurement, found peak OD values between 530 and 540 nm (C. M. Williams et al., 2011). Given small differences in OD values at 540 nm (OD less by ~2% of OD at 534 nm) relative to 534 nm, and given that other studies using the SPV assay for lipid quantification have measured OD at 540 nm (e.g., Cheng et al., 2011; Frings, Fendley, Dunn, & Queen, 1972), we used this wavelength for subsequent quantification of lipids.

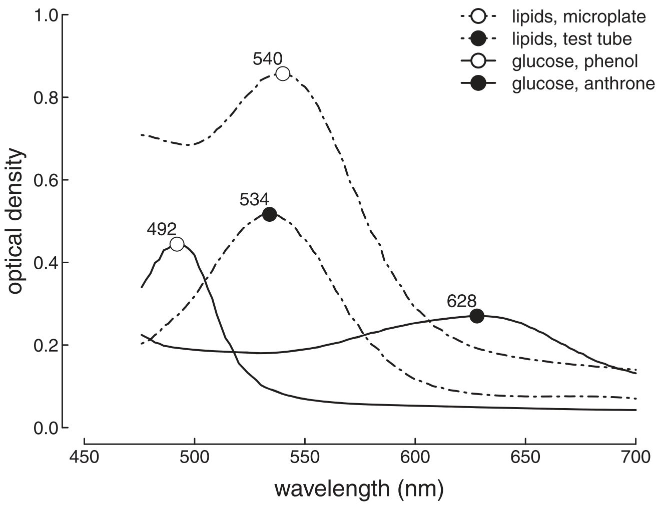

Figure 1. Absorbance scans revealed clear peaks in optical density for lipids and carbohydrates. Absorption spectra of color products from lipid assays (dashed lines) following the microplate (open circle) and test tube (solid circle) methods had optimum wavelengths at 540 nm and 534 nm, respectively. Absorption spectra for carbohydrate assays (solid lines) using the phenol (open circle) and anthrone (solid circle) methods had optimum wavelengths at 492 nm and 628 nm, respectively.

For quantification of carbohydrates using the phenol method, OD peaked at 492 nm, very close to 490 nm reported in the literature (Masuko et al., 2005; Figure 1). Again, given minimal difference in OD values at 490 nm (OD less by 0.6% of OD at 490 nm) and 492 nm, and to maintain consistency with the literature (Masuko et al., 2005), we took all subsequent measurements of OD using the phenol method at 490 nm. The absorbance scan for quantification of carbohydrates using the anthrone method gave a shallow curve with a peak OD at 628 nm (Figure 1), similar to the 625 nm peak (OD less by 0.3% of OD value at 628 nm) found by Van Handel (1985b). We therefore measured OD at 625 nm for measurements of carbohydrate standards and extracts by the anthrone method.

Standard curves

Lipids

We generated standard curves for lipids using a series of standard solutions of soybean (and tripalmitin and triolein for comparison, 0.031 mg/mL to 2 mg/mL) using both microplate and test-tube methods. Overall, the microplate method yielded highly variable and less repeatable OD values for known lipid concentrations. The OD values varied strongly among triplicate measures within a trial (error bars around points in Figure 2a and 2b) and among soybean trials (Figure 2a), particularly at low lipid concentrations. Within soybean trials, one of the standard curves predicted most of the variation in OD values as a function of lipid concentration (Multiple $R^2 = 0.99$; Figure 2a), whereas the other two standard curves had more unexplained variation ($R^2 = 0.65, 0.93$; Figure 2a). Triolein, tripalmitin and 50:50 triolein:tripalmitin curves had similarly moderate R^2 values (0.84, 0.76, and 0.94, respectively) and pronounced variation among triplicate measures within a trial (Figure 2b).

Standard curves generated by the test-tube approach predicted most of the variation in OD values as a function of lipid concentration (R^2 for soybean oil standards $= 0.96$, 0.98, and 0.99, R^2 for TRO $= 0.99$, and R^2 for TRP $= 0.89$, Figure 2c and 2d). Variation in OD values among triplicate measures was also smaller for the test-tube method as compared with the microplate method (compare Figure 2c and 2d with Figure 2a and 2b). Given the limited variation among triplicates and consistent linearity and predictability of standard curves, the test-tube method was chosen as the preferred approach for determination of lipid concentrations.

Carbohydrates

We generated standard curves for carbohydrates using a series of standard solutions of glucose using both phenol and anthrone methods (concentration range $=$ 0.031 to 4 mg/mL). Additional anthrone trials were run for standard concentrations up to 8 mg/mL but, given reaction saturation above about 4 mg/mL (Figure 3) those values are not shown (we recommend sample dilutions to keep measured concentrations below 4 mg/mL). Although both methods gave linear standards curves with high R^2 values (0.96 and 0.99 for anthrone and phenol methods, respectively), the phenol approach had high variability among triplicate measures (see error bars in Figure 3). We therefore used the anthrone method for quantifying carbohydrates.

Quantification of energy components

Lab-reared B. impatiens

The initial wet weight did not differ between *B. impatiens* in the fed (177 ± 33 mg) and starved (173 ± 49 mg) treatment groups ($t_{15} = 0.2$, $P = 0.851$). On average, bees lost 62 ± 23 mg (36% of initial weight) after being starved for ~48 h ($t_9 = -8.6$, $P < 0.001$). This weight reduction was in part due to water loss as starved bees had significantly lower water content than fed bees (starved $=$ 74 ± 25 mg, fed $=$ 116 ± 21 mg; $t_{17} = 4.1$, $P < 0.001$). Starved bees also had significantly lower dry weight than fed bees (starved $=$ 38 ± 11 mg, fed $=$ 61 ± 16 mg; $t_{15} = 3.8$, $P = 0.002$). Parallel declines in water and dry weight meant that percent body water did not differ between treatments (fed $=$ 66 ± 5%, starved $=$ 66 ± 3% of wet weight; $t_{14}=0.2$, $P = 0.867$).

Starved bees had significantly higher proportion of lipids (fed $=$ 0.4 ± 0.1%, starved $=$ 0.5 ± 0.1% of dry weight; $t_{15}=-3.5$, $P = 0.003$) and lower proportion of glycogen (fed $=$ 1.8 ± 0.4%, starved $=$ 0.8 ± 0.1% of dry weight; $t_{10}=7.0$, $P < 0.001$) per mg of dry weight compared to the fed ones. However, proportion of sugars was indifferent across treatment groups (fed $=$ 2.1 ± 0.7%, starved

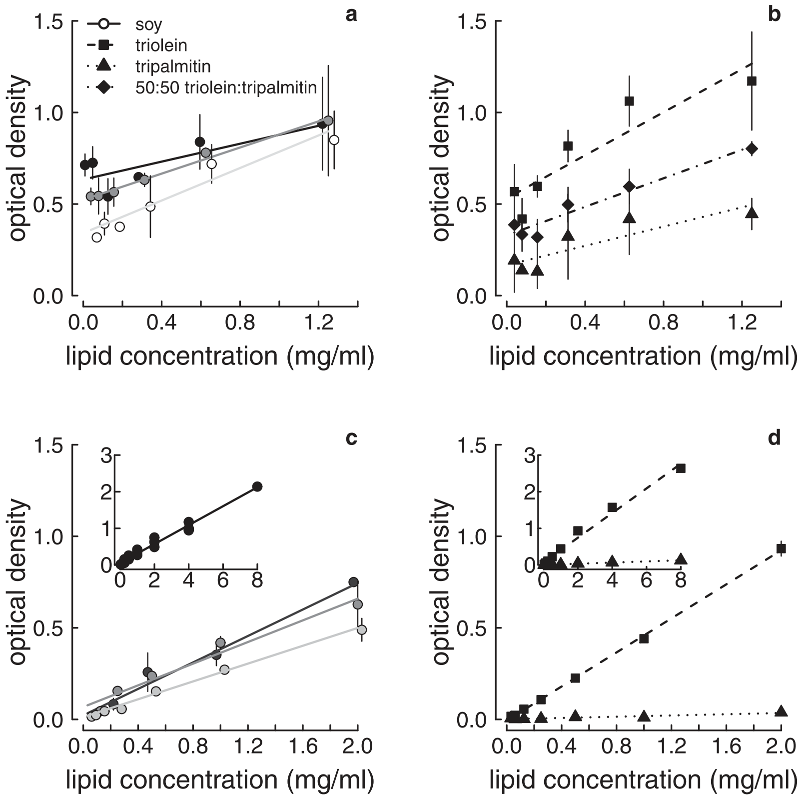

Figure 2. The reliability and repeatability of standard curves for lipid quantification varied with method. Standard curves for known lipid solutions generated by microplate (a, b) and test-tube (c, d) methods for soybean (circles), triolein (squares), tripalmitin (triangles) and 50:50 triolein: tripalmitin mix (diamonds). Points and error bars represent mean and standard deviation of triplicate samples. Three separate runs for soybean oil are represented by grayscale points and lines (a, c). Insets in c and d show extended concentration ranges.

= 1.5 ± 0.8% of dry weight; t_{15}=1.7, $P = 0.104$, Figure 4). The proportion of dry weight made by other body tissues was, on the other hand, higher in starved bees (starved = 95.8 ± 1%, fed = 97.2 ± 0.8%; t_{15}=-3.4, $P = 0.004$). We also found that two of the ten bees in the starvation treatment were still alive after 48 h (and hence were euthanized), but did not differ in energy content from bees that died during the experiment. Because quantities and type of lipids may determine water retention in the body and glycogen storage may play role in water amount (Kaplan & Chaikoff, 1936), we also compared whether the proportion of lipid or glycogen (in addition to the sugars) determined the proportion of water. Water percent increased significantly with increase in proportion of lipid ($F_{1,\ 7}$=11.76, $P = 0.011$, $R^2 = 0.63$) and decreased with increase in proportion of both sugar ($F_{1,\ 7}$=5.0, $P = 0.060$, $R^2 = 0.42$) and glycogen ($F_{1,\ 8}$=56.22, $P < 0.001$, $R^2 = 0.88$) in fed bees, whereas it was indifferent in starved bees (proportion of lipid: $F_{1,\ 7}$=0.08, $P = 0.790$, $R^2 = 0.01$; proportion of sugar: $F_{1,\ 7}$=1.83, $P = 0.218$, $R^2 = 0.207$; proportion of glycogen: $F_{1,\ 8}$=1.786, $P = 0.218$, $R^2 = 0.18$).

Field-collected Bombus huntii

The treatment groups differed significantly in all of the variables measured except for proportion of dry weight made by lipids (Figure 5). We did not measure wet weight of all bees at the beginning of the experiment although we have dead wet weight for all bees. At the end of the experiment, wet and dry weights differed significantly among treatments (ANOVA, $F_{2,27}$=13.5 and 14.6, both $P < 0.001$), with fed bees having significantly higher wet and dry weights than both field and starved bees (Tukey HSD, $P < 0.01$; wet and dry weights of field and starved bees were indistinguishable; $P = 0.143$ and 0.963, respectively). Water content also differed significantly among treatment groups (ANOVA, $F_{2,27}$=10.89, $P < 0.001$), with field bees having significantly higher water percent (67% of dead wet weight) than both fed (58% of dead wet weight) and starved bees (61% of dead wet weight, Tukey HSD, both $P < 0.004$), which were indistinguishable (Tukey HSD, $P = 0.151$).

Proportion of dry weight made by lipids was similar in bees from all three treatment groups (Table 1, Figure 5; $F_{2,\ 22}$=0.198, $P = 0.822$). Proportion of dry weight occupied by sugar and glycogen did not differ between field-collected and starved bees. Both sugars and glycogen in these groups were greatly reduced relative to values for fed bees (Table 1; Figure 5). Eight of ten bees in the starved treatment were dead within 48 h and two of them died within 21 h. One of the bees that died quickly had the lowest recorded proportion of lipid (for *B. huntii*) and very low sugars and glycogen. Similar to lab-reared female *B. impatiens*, field-collected starved male *B. huntii* also had comparably highest dry weight

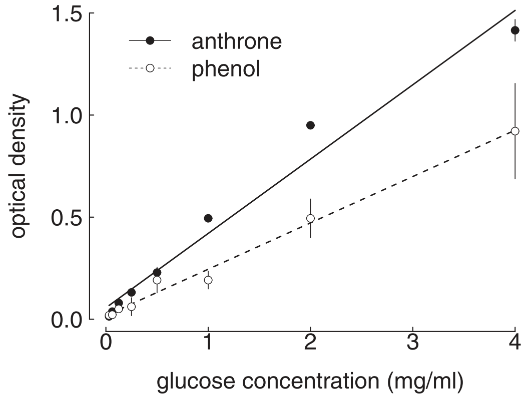

Figure 3. Standard curves for carbohydrates varied between anthrone (solid points and line) and phenol (open points and dashed line) methods. Data points and error bars represent means and standard deviations of triplicate measures.

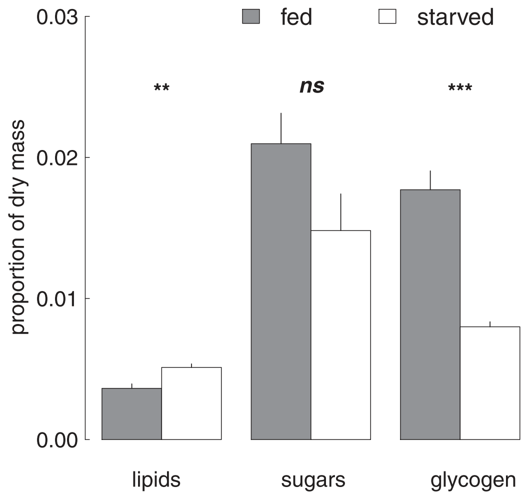

Figure 4. Lab-reared *Bombus impatiens* that were starved for 48 h had reduced dry weight and carbohydrate stores. Mean ± S.E.M. of energy components are given for fed (filled bars) and starved (open bars) bees. Stars indicate significant differences by Welch's two sample *t*-tests (*$P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, respectively).

proportions made of other tissues ($F_{2,\,21}$=25.2, $P < 0.001$, Table 1). Proportion of water in both field and fed bees did not vary with either the proportion of lipids (both $P > 0.160$, both $R^2 < 0.26$), the proportion of sugars (both $P > 0.55$, both $R^2 < 0.50$) or the proportion of glycogen (both $P > 0.15$, both $R^2 < 0.30$) neither did it vary with the proportion of glycogen for starved bees ($P = 0.44$, $R^2 = 0.07$). However, unlike for the *B. impatiens*, proportion of water in starved bees decreased significantly with an increase in either the proportion of lipid ($F_{1,\,6}$=12.7, $P = 0.012$, $R^2 = 0.68$) or the proportion of sugars ($F_{1,\,7}$=5.1, $P = 0.057$, $R^2 = 0.42$).

Discussion

Extraction and quantification methodology

For bumble bees, the extraction procedure described by Van Handel (1965) and Van Handel & Day (1988), as

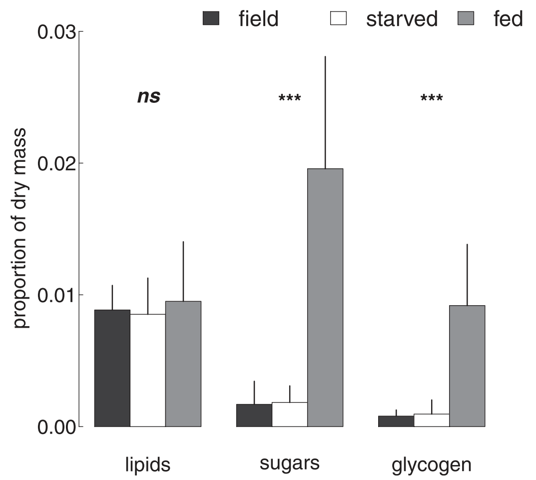

Figure 5. Field-collected, starved, and fed male *Bombus huntii* differed in energy stores. Mean ± s.e.m. of energy components are given for field-collected (gray), starved (white) and fed (black) bees. Stars indicate significant differences by Welch's two sample *t*-tests (*$P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$) (see Table 1).

modified by Benedict (2007) causes the glycogen precipitate to get trapped in the tissue residue. The modified extraction procedure described here is straightforward and allows for proper extraction and separation of energy components from bee tissue in just a few steps with minimal possibility of losing glycogen.

Our analyses also suggest that the test tube method (Van Handel, 1985b) is preferable to the microplate method (Cheng et al., 2011) for lipid quantification in bumble bees. The microplate method is appealing because it is potentially high-throughput, with small reagent volumes ($\leq$ 100 μL per sample) and minimal transfer of solutions between reaction vessels. However, the microplate approach requires the use of organic solvents which appear to corrode the polystyrene microplate wells, leading to inconsistencies and pronounced variation in OD readings (Figures 1a, 2a, 1b, 2b; see C. M. Williams et al., 2011). Microplates resistant to organic solvents are expensive and less convenient as they must be thoroughly cleaned between uses. The test tube approach requires larger reagent volumes, but produced reliable and reproducible measurements (Figure 2c and 2d).

One limitation of the SPV assay is that the colorimetric reaction depends on the presence of carbon-carbon double bonds. Therefore, the method cannot detect saturated lipids (Moran & McAlister, 2009) and reactivity with polyunsaturated fatty acids will decrease with increasing unsaturation (Knight et al., 1972; C. M. Williams et al., 2011). Accordingly, this method may lead to systematic errors of small magnitudes for samples with significant amounts of mono- or di-acylglycerides or if the fatty acid composition varies across treatment groups. With these limitations in mind, the SPV assay can be a reliable, straightforward, and high-

Table 1. Body weight, proportion of dead weight made of water, and proportions of dry weight of energy substrates and other tissues for field-collected, starved, and fed *Bombus huntii* males. All values are mg (or %) ± SD. *F*- and *P*-values are from full-model ANOVAs, with superscript letters indicating significant differences among treatments by Tukey HSD *post hoc* comparisons.

Parameters	Field	Starved	Fed	*F* (df)	*P*
Wet mass (mg)	97.6 ± 9.4^{a}	80.8 ± 10.2^{a}	125.0 ± 30.2^{b}	13.5 (2, 27)	<0.001
Dry mass (mg)	32.6 ± 3.0^{a}	31.4 ± 3.7^{a}	53.0 ± 16.8^{b}	14.6 (2, 27)	<0.001
Water (%)	66.6 ± 1.1^{a}	61.0 ± 2.3^{b}	58.1 ± 5.5^{b}	16.0 (2, 27)	<0.004
Lipid proportion (%)	0.9 ± 0.2^{a}	0.9 ± 0.3^{a}	1.0 ± 0.5^{a}	0.2 (2, 22)	0.822
Sugar proportion (%)	0.2 ± 0.2^{a}	0.2 ± 0.1^{a}	2.0 ± 0.9^{b}	34.9 (2, 24)	<0.001
Glycogen proportion (%)	0.1 ± 0.0^{a}	0.1 ± 0.1^{a}	0.9 ± 0.5^{b}	28.6 (2, 25)	<0.001
Other (%)	98.9 ± 0.3^{a}	98.9 ± 0.4^{a}	96.3 ± 1.4^{b}	25.2 (2, 21)	<0.001

throughput method (C. M. Williams et al., 2011). For the bees measured in this study (Figures 4 and 5), even though starvation led to depleted energy content, we expect little variation in fatty acid composition among treatments in such a short time frame, as starvation does not change fatty acid composition for a diversity of insect taxa (Canavoso, Bertello, de Lederkremer, & Rubiolo, 1998; Haubert, Häggblom, Scheu, & Ruess, 2004; e.g., Wlodawer & Wiśniewska, 1965). As such, application of the SPV assay for quantification of lipids is reliable.

We found the anthrone method to be superior to the phenol method for quantification of carbohydrates in bumble bees. In contrast to the phenol method, the anthrone approach gave consistent measures among triplicates (Figure 3). The anthrone approach is excellent for analysis of glucose-based carbohydrates such as starches, modified starches, or dextrins (Laurentin & Edwards, 2003) and does not require previous hydrolysis of polysaccharides into component sugars (Raunkjaer, Hvitved-Jacobsen, & Nielsen, 1994). However, because anthrone reacts differently with different carbohydrate moieties (Raunkjaer et al., 1994), although using a single sugar as a suitable standard may be useful for generalizations, it has its limitations for a sample containing a mixture of carbohydrates. Further, the anthrone-sulfuric acid reagent is unstable so must be prepared every three to four days (even when stored in dark and at 4 °C).

Energy components in Bombus

As expected from studies in other insects (Fadamiro & Heimpel, 2001; Gibbs & Reynolds, 2012; Marron et al., 2003; Wlodawer & Wiśniewska, 1965), starvation altered quantitative nutrient composition of both lab-reared *B. impatiens* and field-collected *B. huntii* (Figures 4 and 5). Similar to previous studies (reviewed in Arrese & Soulages, 2010; Mwangi & Goldsworthy, 1977; Ziegler, 1991), we found an increase in lipids and a strong depletion of glycogen in starved relative to fed bees. Those studies have shown that starvation leads to a depletion of hemolymph carbohydrates and an increase in hemolymph lipids, which are mobilized from the fat body. Because our measurements were for the whole bees, we were unable to distinguish between lipid content in the hemolymph and fat body, but given the sensitivity and efficacy of this approach, future studies could separately quantify energy components for different body compartments of bees. The observed increase in proportion of other body tissues in starved bees may, in part, be because of depletion of the energy components, which might have led to the increase in percentage of these other tissues.

Field-collected male *B. huntii* were indistinguishable from starved bees in terms of lipids, sugars, and glycogen content (Figure 5), suggesting that foraging males are living on the edge in terms of energy resources. Further, when male *B. huntii* were fed, they not only had higher sugar and glycogen levels but also had higher lipids. The supplied sugar water had no lipids, suggesting that these male bees converted sugars to lipid stores during the 48-h experiment (e.g., Arrese & Soulages, 2010).

Likely, in part due to feeding history, bees varied in how long they survived the starvation treatment. In particular, field-collected male *B. huntii* died at least 24 h before lab-reared female *B. impatiens*. The total energy stored for field-collected *B. huntii* was similar to that of starved bees, indicating that these bees were already deprived of energy at capture and therefore likely to be more vulnerable to food deprivation. Because diet history may substantially affect organism's body composition (e.g., Karowe & Martin, 1989), the diet prior to the experiment may also have affected the survival duration.

In general, female insects tend to have relatively more lipids than do males (Ballard, Melvin, & Simpson, 2008; Lease & Wolf, 2011), in large part due to differences in reproductive demands. In contrast, we found that male *B. huntii* had relatively more lipids (~0.9% of dry weight for all treatments) than did female *B. impatiens* (~ 0.4% of dry weight). If these differences hold for comparisons between males and females of the same bumble bee species, it would suggest that these social insects do not match the general pattern found in other insects. Perhaps because bumble bee workers do not provision or lay their own eggs, lipid demands are reduced. Another potential cause may be the differences in the background of the bees compared in this study as the female *B. impatiens* were reared in the lab for many generations and male *B. huntii* were wild.

We also compared the relationship between proportion of water in bee body and either lipids or carbohydrates. Whereas the proportion of water was directly proportional to the proportion of lipids and inversely proportional to the proportions of both sugars and glycogen in fed *B. impatiens*, it did not vary in either starved *B. impatiens* or in *B. huntii* exposed to any treatment. As simple this relationship is, the relationship between proportions of water and lipids was complex and varied in directions for the species of bees. Firstly, the proportions of water and lipid were directly proportional in fed *B. impatiens*. Secondly, proportions of water and lipid were inversely related in starved *B. huntii*. These differences in our observations might be in part due to the differences in species, sex and background of the bees (*B. impatiens* was lab-reared and *B. huntii* were field-collected).

All values reported in this manuscript were the average of triplicate readings for both standards and bee samples. Whereas multiple assays were performed for the standards to test the repeatability of the methods, we did not perform multiple extraction and quantification from a single bee or a pool of bees. Future studies could perform multiple extraction and quantification in samples too to verify the repeatability of the method. The straightforward, rapid, and inexpensive method for extraction and quantification of energy stores described in this paper should prove useful in assessing nutritional status of bumble bees (and potentially other bees) in both applied and natural settings, particularly in the context of ongoing threats to these critical pollinators (Cameron et al., 2011).

Acknowledgments

The authors thank R. Peesara (Department of Chemistry, UW), A. Navratil, and C. Martinez del Rio (Department of Zoology and Physiology, UW) for advice on analyses and use of equipment, the UW Robert A. Jenkins Microscopy Facility for instrument support, and J. Vogt for help with lab-reared bees. The authors also thank Muna Fuyal, Tshering Ongmu Sherpa, Pravin Pokhrel and Shashank Jha for critically reviewing this paper.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by a University of Wyoming (UW) Arts & Sciences Summer Independent Study Award, 2012 and a UW–National Park Service Research Center Grant [grant number GRTE-2012-SCI-0036], [Study number: GRTE-00219], [Study period 2012-2013].

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