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SHORT COMMUNICATION

A handbook for uncovering the complete energetic budget in insects: the van Handel's method (1985) revisited

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Abstract. Insects comprise relevant biological models for investigating nutrient acquisition and allocation processes in the context of life-history ecology and evolution. However, empirical investigations are still partly limited by the lack of availability of simple methods for simultaneously estimating the four major energetic components (i.e. lipids, free sugars, glycogen and proteins) in the same individual. In the present work, we validate a fast, reproducible and cheap method for overcoming this problem that uses different solvents successively. First, proteins are solubilized in a phosphate-lysis buffer and then quantified according to the classical Bradford assay procedure. In a second step, a chloroform–methanol mixture is added to the aqueous phase, which allows assay of the total lipid fraction, as well as the free sugars and glycogen in the same insect homogenate. In addition, a micro-separation procedure is adapted to partition the total lipids into neutral (mainly stored lipids) and polar (mainly structural lipids) components. Although these assays are conducted sequentially in the same individual, the sensitivity of our method remains high: the estimated amount of each energetic compartment does not differ from that obtained with former, partial methods. Our method should thus largely improve our knowledge about nutrient acquisition and allocation among insects not only in laboratory-reared individuals, but also in animals caught in the wild. Descriptions and recommendations are given at each step of the protocol to adapt the procedure to various insect species. Finally, to prevent misinterpretation of data generated in accordance with this protocol, the limits of our method are discussed in the light of life-history studies.

Key words. Carbohydrates, colourimetric assays, energetic reserves, glycogen, proteins, lipids, nutrient assays, resources acquisition and allocation.

Introduction

Insects comprise relevant biological models for investigating nutrient acquisition and allocation processes in an ecological and evolutionary perspective, using both field and laboratory approaches (Ellers & van Alphen, 1997; Olson *et al*., 2000; Casas *et al*., 2003, 2005; Lee *et al*., 2004, 2006). To assess nutrient contents of individuals, most ecological studies rely on the colourimetric methods first proposed by van Handel. These assays were initially developed to quantify both glycogen and free sugars in one individual (van Handel, 1985a) and lipids in another individual (van Handel, 1985b). More recently, these methods have been modified to simultaneously estimate, in the same individual, the three main categories

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of nutrients: glycogen, free sugars and lipids (Olson *et al*., 2000; Giron *et al*., 2002; Fadamiro & Chen, 2005; Chen & Fadamiro, 2006). These methods are extensively used to determine the energy stored in single insects given their cheap, easy, rapid and efficient characteristics. They notably allow the incorporation of greater physiological realism into behavioural ecology and population dynamic studies on parasitoid insects (Rivero & Casas, 1999). They are also used to link the nutrient levels and/or the feeding source of an insect (e.g. nectar versus honeydew) to its longevity or egg load (Olson *et al*., 2000; Lee *et al*., 2004; Lee & Heimpel, 2008). However, despite these attempts, the current available methods present some shortcomings that may constrain the experimental design and/or relevance of studies investigating life-history trajectories of insects. In most cases, the total lipid fraction is inadequately used as a proxy for stored lipids, which mainly consist of triglycerides. Indeed, the proportion of triglycerides in the total lipids is known to fluctuate considerably according to both the developmental stage of the insect and the season (Lee *et al*., 1975). Therefore, estimating the stored lipids requires removing the structural polar lipids (i.e. the phospholipids that form a negligible part of the fat reserves) from the total lipids. Proteins may also represent an important energetic source in insects (Hahn *et al*., 2008); for example, to fuel flight (Suarez *et al*., 2005), egg production (Bernstein & Jervis, 2008) and diapause (Hahn & Denlinger, 2007). Specific colourimetric methods are currently used to measure the total amount of aqueous buffer-soluble proteins stored in the insect tissues (Gornall *et al*., 1949; Lowry *et al*., 1951; Bradford, 1976). However, proteins do not share the same affinity with solvents compared with other stored compounds (i.e. proteins are best suspended in aqueous buffers, whereas lipids and carbohydrates are dissolved in organic solvents); thus, researchers are prevented from extracting all the nutrient categories present in the insect tissues by a single solvent. There are some reported studies that attempt to assay proteins together with other nutrients (Zhou *et al*., 2004), although the procedure is often complex and notably involves specific steps (such as resolubilization) that are likely to lose some compounds before the start of the assay. This might explain why the energetic budget of an insect organism is still commonly extrapolated from partial data obtained on several individuals expected to be in the same physiological state (Brent *et al*., 2011). Such limitations require insects to be raised in highly controlled laboratory conditions, thereby precluding field studies of insects caught in the wild with unknown and variable life trajectories.

The main objective of the present study is to set-up a reliable and reproducible biochemical protocol to estimate the complete energetic budget of a single insect and to provide nonspecialist users with some information and recommendations about this method for further application on diverse insect species. The proposed method assays the amount of free sugars, glycogen, proteins and total lipids in single individuals weighing only a few milligrams. In addition, a finer-scaled analysis that partitions the neutral and the polar lipid fractions is suggested. Neutral lipids are essentially (but not only) triglycerides and may be used as a relevant proxy for lipid reserves in most insect species, whereas polar lipids are mainly phospholipids that are major structural components of the biological membranes (Canavoso *et al*., 2001). Finally, this method is designed for microplates, which allows simultaneous measurements on large insect sample sizes, thereby improving the reproducibility of the measures by minimizing the 'run to run' error.

This method is tested on a hymenopteran parasitoid *Venturia canescens* Gravenhorst (Hymenoptera: Ichneumonidae) because individuals can be reared easily under standard laboratory conditions and thus display fairly similar nutrient content on emergence (Pelosse *et al*., 2007, 2010). The method is validated by comparing the results with data obtained using two separate, well-established methods: one assaying the protein content and the other one assaying lipid, glycogen and free sugars contents.

Materials and methods

Insect

Venturia canescens is a mediterranean endoparasitoid that parasitizes lepidopteran larvae (Salt, 1976). Adults feed on sugary food and are unable to synthesize lipids from sugar-feeding (Casas *et al*., 2003; Desouhant *et al*., 2005, 2010). Females produce small, hydropic and yolk-deficient eggs (Le Ralec, 1995). The females were reared from a parthenogenetic thelytokous strain founded in summer 2008 from wild-caught females (North: 44°58′34″, East: 4°55′66″; INRA Gotheron, France). Larvae of the pyralid moth *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae) fed on wheat semolina were used as hosts. Both hosts and parasitoids were reared under an LD 12 : 12 h photocycle at 25 ± 1 °C and 70 ± 5% relative humidity.

General design

The method evaluated is designed to measure, in the same organism, proteins, carbohydrates, glycogen and lipids (and, among them, neutral lipids) in 96-well microplates. Unlike previous methods, insects are first crushed into an aqueous lysis buffer to extract and quantify their protein content with the Bradford assay. In a second step, a chloroform: methanol (1 : 2 v/v) solution is added to reach the proportions 2 : 5 : 10 v/v/v of water, chloroform and methanol, respectively. This step allows the extraction of total lipids, carbohydrates and glycogen that are further assayed with the van Handel's methods (van Handel, 1985a, b) (Fig. 1). To validate this new method, estimations of the four energetic compartments were made versus those of conventional, former methods (see Introduction).

Sample preparation

Biochemical experiments were carried out on 64 newly-emerged females considered to have similar levels of nutrients, with each of these being randomly assigned to one of three groups. In the first group, referred to as the 'New method'

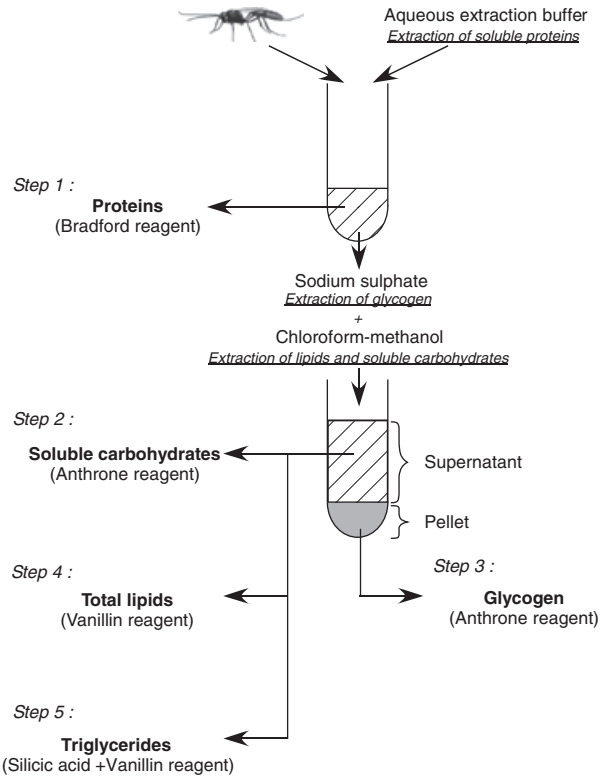

Fig. 1. General principle and successive steps of the method used to quantify the amount of proteins, total lipids, total sugars and glycogen in single individuals. The properties of the solutions added at the different steps are underlined and the reagents used for the determination different energetic contents are given in parenthesis.

($n = 21$), the main energetic compartments [i.e. proteins, total lipids (and among them, neutral lipids), total sugars and glycogen] were quantified for each female. In the second group, referred to as the 'Bradford method' ($n = 20$), only proteins were quantified with the Bradford colourimetric assay. In the third group, referred to as the 'van Handel method' ($n = 23$), total lipids, total sugars and glycogen were quantified in accordance with the method of Giron *et al*. (2002). Each group consisted of newly-emerging female parasitoids that were immediately and individually frozen dry at -20 °C in 2-mL Eppendorf vials. To correct for a possible effect of body mass on energetic reserves, each wasp was weighed to the nearest 0.1 mg. The body mass of wasps was homogeneous among the three groups ($F_{2,61} = 0.39, P = 0.68$).

In any colourimetric assay, it is essential to set up precise, multipoint standard curves to check for linearity within a range of concentrations that should start below the lowest sample value and end beyond the highest one, as well as to gauge the repeatability and the reproducibility of the procedure. These conditions were first verified in a preliminary experiment by setting up seven independent repeats of the standard curves, each with eight distinct points corresponding to known concentration values (including the blank). For each type of assay, we checked that the repeated standard curves fitted with a linear regression (range of R^2: protein curves = 0.96–0.99; carbohydrate curves = 0.95–0.99; lipid curves = 0.92–0.98) and presented similar slopes (analysis of covariance: interaction between the concentration of the standard and the curve series: protein curves, $F = 1.70$, $P = 0.20$; carbohydrate curves, $F = 0.08$, $P = 0.77$; lipid curves, $F = 0.96$, $P = 0.33$). Furthermore, in the present study, the standard curves have been systematically duplicated in each microplate and measured simultaneously to the insect samples aiming to take into account the variability possibly occurring across microplates that could be a result of slight differences in the incubation time, the temperature or even the quality of the reagent used.

The method was adapted to be carried out on microplates, which implies the use of small volumes. To reduce the sources of experimental (mostly pipetting) errors, the use of appropriate materials, particularly low-retention tips and an electronic multichannel pipette, is recommended.

New method: measurement of the energy reserves in whole insect

Each insect was individually placed into a 2-mL Eppendorf tube containing a stainless steel bead and 180 μL of aqueous lysis buffer solution [100 mM KH_2PO_4, 1 mM dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4] and was crushed by shaking the tube for 30 s at 25 Hz (Tissue Lyser; Qiagen, Valancia, California). The phosphate buffer is well known for its solubilizing properties. Because it chelates divalent metals ions, EDTA inhibits metalloproteases and prevents from metal ions-catalysed oxidations, which limits oxidative damage to proteins. DTT is a reducing agent that also decreases the oxidative damage to proteins and prevents the disulphide bonds between cysteine residues of proteins. The concomitant presence of EDTA and DDT considerably improves the solubility and stability of proteins in the lysis buffer (but see also Discussion). Furthermore, the use of a high-speed bead-beating apparatus is crucial at this step of the protocol not only to secure efficient disruption of insect tissues, but also to optimize lipid dispersion and emulsification in the aqueous phosphate buffer. Although the addition of detergent (e.g. Triton X) and/or emulsifier (e.g. Tween) in the phosphate buffer would undoubtedly lead to a better dispersion of the lipids, these molecules are incompatible with the subsequent colourimetric assays and thus are not relevant in the present study. This first step enables efficient cell lysis and reasonable solubilization of proteins at the same time as avoiding interference with further colourimetric assay. The volume of aqueous lysis buffer solution used per insect (180 μL) is appropriate for organisms weighing up to 15 mg. For bigger insects, this volume should be increased to ensure the solubilization of proteins and the dispersion of lipids (see below).

Protein content determination

Each homogenate was subjected to low-spin centrifugation (180 ***g*** at 4 °C), which allowed gentle sedimentation of cuticle

and cell debris that otherwise would alter the clarity of the sample. In accordance with the manufacturer's instructions, 2.5 μL of each supernatant was transferred into a 96-well microplate, together with 250 μL of Bradford micro-assay reagent (B6916; Sigma, France). Samples were incubated at room temperature for 15–20 min (the protein–dye complex is stable up to 50 min). At this stage, if the lipids do not disperse homogeneously into the buffer and form a thin surface slick on the top of the homogenate, caution must be taken not to remove them when pipetting a 2.5-μL volume for protein assay. For protein determination, the Bradford assay was chosen because it is well-known for providing reliable results (Kruger, 2002). This Coomassie-based protein assay is compatible with most salts, reducing substances and metal-chelating agents, and affords low interference with nonprotein substances present in the sample. Protein concentration was determined spectrophotometrically at 595 nm (Xenius SAFAS, Monaco) using a dilution-series of bovine serum albumin dissolved into the same buffer as the standard. Before reading, microplates are gently shaken in the microplate reader (10 Hz for 3 s) to disrupt protein-dye aggregates.

Carbohydrate content determination

To dissolve all the carbohydrates, 20 μL of 20% sodium sulphate solution (Na_2SO_4; Sigma) was then added to the homogenate, as well as 2.5 μL of the extraction buffer solution (see above) to reach a final solution of 0.2 mL of 2% Na_2SO_4 (van Handel, 1965; van Handel & Day, 1988). This solution was then mixed with 1500 μL of a chloroform–methanol solution (1 : 2 v/v) to solubilize the total lipids, as well as the water-soluble carbohydrates (van Handel, 1965; van Handel & Day, 1988). After vigorous vortexing, each sample was centrifuged for 15 min at 180 ***g*** and 4 °C to remove glycogen from the supernatant that was transferred into a new tube for subsequent analysis. The pellet was kept for further determination of the glycogen content.

All the carbohydrates, including glycogen and the soluble carbohydrates, were determined using the classical colourimetric method based on anthrone reagent (van Handel, 1965) in a two-step procedure (see below). This colourimetric technique is an accurate qualitative test where a blue–green colour appears when anthrone reagent specifically binds to mono-, di- or polysaccharides present in the homogeneous suspension. This technique has been used considerably subsequent to the original publication of the method in the middle of the 20th Century. However, the instability of anthrone over time requires preparing only low volumes of this reagent at a time and using it within 2–3 days. Fresh anthrone reagent was prepared by mixing anthrone powder (Fluka 10740; Fluka, Switzerland) with sulphuric acid 70%, so as to reach a final concentration of 1.42 g L^{-1}. After preparation, the glass bottle was then wrapped in aluminium foil to be safe from light until use. For carbohydrate determination and all subsequent assays, a flat-bottom 96-well borosilicate microplate (730.009-QG; Hellma Analytics, Germany) was used to perform the bioassay because the organic solvents used are incompatible with standard polystyrene microplates. In a first step, 150 μL of the initial supernatant of each insect sample was transferred into a microplate well and was evaporated for approximately 30 min at room temperature until a volume of approximately 10 μL was reached. Then, 240 μL of anthrone reagent was added to each well and the plate was incubated for 15 min at room temperature. The microplate was then carefully covered and heated 15 min at 90 °C in a water bath, after which the absorbance of the samples was read at 625 nm using D-glucose (Fluka 49140) as the standard. This allows determination of the total water-soluble carbohydrates (van Handel, 1965, 1985a).

It is worth noting that cold anthrone reaction (i.e. reading the optical density before incubating the sample at 90 °C) may be used to determine fructose content, whereas the determination of total carbohydrates requires heating the sample, as described above (van Handel, 1967; Fadamiro & Chen, 2005). This method has already been used to analyze the feeding source of parasitoids; notably to discriminate nectar and honeydew (Lee *et al*., 2006; Wyckhuys *et al*., 2008). In a second step, the glycogen contained in each insect was assayed by twice washing the pellets containing sodium sulphate and bounded glycogen, using 2 × 400 μL of 80% methanol (Fig. 1). The washing step included vigorous vortexing followed by centrifuging for 5 min at 16 000 ***g*** and 4 °C, and the supernatant was removed with a micropipette. One millilitre of anthrone reagent was then added to the pellet, followed by 15 min of incubation at 90 °C in a water bath. Samples were cooled on ice to stop the reaction and filtered on low-protein binding membranes (polyvinylidene fluoride; d = 0.45 μM , Durapore; Millipore, Billerica, Massachusetts). The glycogen content was then determined by measuring the absorbance of the sample at 625 nm with glucose as the standard.

Lipid determination

First, the total amount of lipids in each insect was determined in accordance with the vanillin assay procedure (van Handel, 1985b) using triolein (92860; Sigma) as the standard. Vanillin assay was recently confirmed to be a high-throughput technique providing very accurate data for the determination of lipids in insects (Williams *et al*., 2011). Vanillin reagent was prepared by mixing vanillin (V2375; Sigma) with ortho-phosphoric acid 68%, reaching a final concentration of 1.2 g L^{-1}. Vanillin reagent is stable for several weeks if stored safe from light until use. In the present study, the vanillin reagent was stored in a glass bottle wrapped into aluminum foil and used within 1 week after preparation. For the assay, 100 μL of the supernatant was transferred into a borosilicate microplate well and heated at 90 °C until complete solvent evaporation. Ten microlitres of 98% sulphuric acid was then added to each well and the microplate was incubated at 90 °C for 2 min in a water bath. After cooling the microplate on ice, 190 μL of vanillin reagent was added to each well. The plate was homogenized, incubated at room temperature for 15 min and its absorbance was measured spectrophotometrically at 525 nm.

In a second step, the amount of neutral lipids present in the insect was assayed. This was performed by transferring 500 μL of the remaining supernatant into a new tube, which was then heated dry at 90 °C until complete evaporation of the solvent. One millilitre of chloroform was added to re-solubilize the lipids. In each sample, 200 mg of dry silicic acid (Sila200; Sigma) was added in excess to specifically bind all the polar lipids present in our preparations (van Handel, 1965; Zera & Larsen, 2001; Fig. 1). The mixture was shaken vigorously and centrifuged for 10 min at 180 ***g*** and 4 °C to remove the silicic acid and the bounded polar lipids. Measuring the total lipids contained in a sample free of polar lipids provided a reliable measure of the neutral lipid concentration, which is a proxy for triglyceride content (van Handel, 1965; Zera & Larsen, 2001). The neutral lipid content was therefore measured in a 100-μL aliquot of the final supernatant using the same protocol. This method was validated by testing whether silicic acid specifically binds all the polar lipids at the same time as avoiding neutral lipids. Indeed, according to the quality/dryness of the silicic acid and to the amount of lipids in the sample, the amount of silicic acid has to be adjusted to ensure optimal extraction of polar lipids. Silicic acid eventually has to be dried in an oven (200 °C during one night) and/or to be stored together with desiccant until use. Before applying such protocol to any new insect species, a preliminary validation of the method is recommended by making a known standard of either neutral or polar lipids. In the present study, this protocol was valiadated using two distinct standardized solutions of chloroform–methanol (1 : 2 v:v), one containing a mix of phospholipids (azolectine, 18811145; Sigma) and the other one containing triglycerides (triolein, 92860; Sigma), both at a known concentration (0.4 $\mu g\ \mu L^{-1}$) that corresponds to the mean amount assayed on *V. canescens* females.

Statistical analysis

Each energy compartment was analyzed with a generalized linear model assuming a gamma distribution of the residuals (inverse link) to test the effect of the method. To account for size effect, body mass was always introduced as the first explanatory variable. All analyses and graphics were performed with the R statistical package, version 2.12.0 (R Development Core Team; http://www.r-project.org/).

Results and Discussion

Using the integrated method described in the present study, different solvents are successively employed to sequentially extract and quantify proteins, lipids and carbohydrates in a single individual. Proteins are first extracted in aqueous lysis buffer and assayed in accordance with the classical Bradford procedure (Bradford, 1976). In a second step, the addition of a chloroform–methanol mixture to the aqueous phase enables the total lipid fraction, as well as the free sugars and glycogen, to be assayed in the same insect homogenate in accordance with van Handel's method (van Handel, 1985a, b; van Handel & Day, 1988). Unlike previous colourimetric assays, this method allows an estimation (in the same insect) of the amount of energy stored in the four main energetic compartments: proteins, lipids, sugars and glycogen. Although these assays are conducted successively in the same individual, the sensitivity of this sequential method remains high: the amount of energy in each compartment, estimated using this method, does not significantly differ from that estimated by traditional methods (proteins: $\chi^2 = 0.05$, d.f. $= 1$, $P = 0.09$; lipids: $\chi^2 = 0.018$, d.f. $= 1$, $P = 0.53$; total free sugars: $\chi^2 = 0.0002$, d.f. $= 1$, $P = 0.97$; glycogen: $\chi^2 = 0.18$, d.f. $= 1$, $P = 0.33$; Fig. 2).

Furthermore, the method successfully separates the neutral lipid (i.e. mainly the stored lipids) from the polar lipids. Approximately 94.6% (range: 92.5–96.6%) of the polar lipids are removed, on average, from the standardized solution of known concentrations of phospholipids, whereas 79.9% (range: 68.0–88.5%) of the neutral lipids remain in the standardized solution of triglycerides. The mean ± SD estimation of neutral lipids content provided by this method on a *V. canescens* female is $31.19 \pm 8.79\ \mu g\ mg^{-1}$, which corresponds to approximately 39% of measured total lipids. If the proportion of neutral lipids removed during the micro-separation is taken into consideration, the corrected amount of neutral lipids reaches approximately 50% of the total lipid content in the studied model. This finding thus shows that a relevant estimation of the stored lipid content in insects requires the removal of the phospholipid fraction. This method might open new research perspectives on lipid metabolism and its implication in the nutritional ecology of insects (Visser *et al*., 2010).

Even if the method proposed in the present study could be successfully applied to any insect species for ecophysiological studies, it is necessary to remain cautious when interpreting the data obtained in terms of nutrient allocation at the organismal-level pattern. Several weaknesses are noted with this new method. First, to determine the protein content of the insect with the Bradford method, a lysis buffer with high solubilizing properties is used, although some proteins may fail to dissolve in this buffer. Indeed, the capacity for a buffer to dissolve proteins not only strongly depends on its composition (i.e. presence of detergent, ionic strength) and pH, but also on the intrinsic composition of amino acids in the proteins and their location within the protein (e.g. the presence or absence of highly hydrophobic amino acids at the surface of the proteins). Because some unsolubilized proteins may sediment, it is unlikely that the proteins assayed in the supernatant quantitatively reflect the absolute total protein content of the insect. Furthermore, because the whole insect is crushed in the buffer, the cuticle debris has to be removed through gentle centrifugation, which might also remove the proteins with the heaviest molecular weights. It is suggested that centrifugation should be avoided whenever possible (i.e. if the sample is free of debris). To our knowledge, there is no assay available that is uniformly sensitive to all protein types (with respect to amino acid composition). As an example, the Bradford reagent reacts primarily with arginine, lysine and histidine amino acids, and the assay is known to be very sensitive to pure bovine serum albumin (which is used as a standard), probably much more

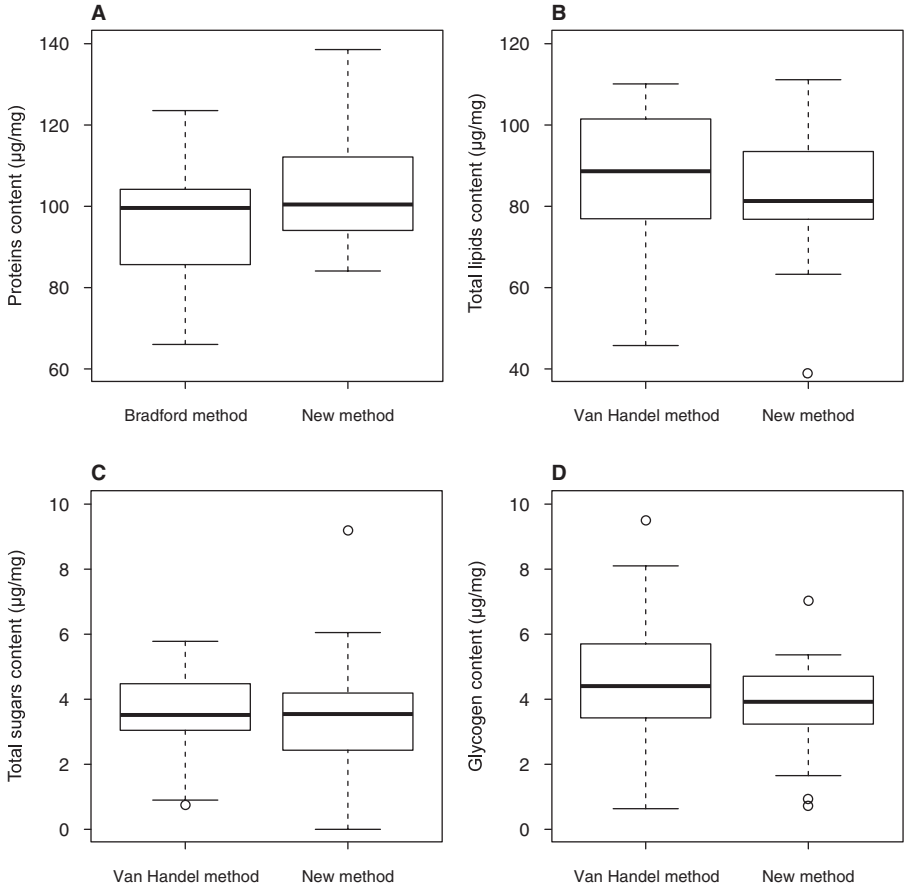

Fig. 2. Boxplots of proteins (A), total lipids (B), total sugars (C) and glycogen (D) amounts determined in female *Venturia canescens* in accordance with the partial protocols or the new extraction protocol. Data are expressed in equivalent bovine serum albumin (A), triolein (B) or glucose (C, D), and are corrected for the mass of the insect that had a significant effect on the four energy compartments (proteins: $\chi^2 = 0.93$, d.f. $= 1$, $P < 0.0001$; total lipids: $\chi^2 = 1.30$, d.f. $= 1$, $P < 0.0001$; total sugars: $\chi^2 = 3.71$, d.f. $= 1$, $P = 0.0001$; glycogen: $\chi^2 = 4.25$, d.f. $= 1$, $P = 0.0001$). Boxplots: horizontal bold line, median; box, lower and upper quartiles; dashed lines, 95% confidence interval. Bradford method, $n = 21$; Van Handel method, $n = 23$; new method, $n = 21$.

so than the assortment of proteins present in our insects. For all these reasons, the protein amount estimated with such a protocol must be interpreted cautiously but could, however, be used efficiently when performing comparative analyses.

The method is also designed to estimate the stored lipids present in the insect. Because the total lipid content obtained with the vanillin assay may not accurately reflect such a category of lipids, we describe the adaption of a micro-separation method that splits the neutral versus the polar lipids. Indeed, neutral lipids are mostly triglycerides (stored in the fat body and used to fuel energy-demanding activities), whereas a residual fraction comprises mono- and diglycerides, as well as free fatty acids. A third fraction of the neutral lipids is composed of non-energetic molecules, such as long chain cuticular and/or wax hydrocarbons, free sterols and sterol esters. Although their relative abundance may be negligible compared with the stored lipids in many insect species, high-performance liquid chomatography or thin-layer chromatography should be used to confirm this assertion in any unknown insect species. It should also be noted that, because some of the neutral lipids are removed during the micro-separation processes, our method may primarily be used to perform comparative analyses rather than to obtain the absolute lipid budget.

The method described in the present study is cheap, easy to use without specific technological devices, and is shown to be sufficiently sensitive and reliable for estimating the total energetic budget of organisms weighing only a few milligrams, and also allows quantification of the main energetic storage compartments in a single individual. Another important aspect of the method is that only a very small amount of biological material is required to assay the different energetic components in insect tissues. Therefore, the method can be advantageously performed at a finer-scale to determine the energetic value of specific organs. As an example, the energy content of the ovarian tissue can be assayed separately from the rest of the body. This is particularly relevant for setting apart the stored versus the reproductive energy components of insects and accessing more finely the energetic resources allocated to a particular key ecological function, such as reproduction. Moreover,

it is designed for analyses on microplates, which considerably lowers artefact errors by allowing the simultaneous assay of numerous individuals (Cheng *et al*., 2011; Williams *et al*., 2011), although it should be noted that the sensitivity of pipetting errors may increase as a result of scaling down the volume of the assay.

This method should significantly improve studies of the acquisition of nutrients and their allocation towards survival and various ecological functions not only in laboratory-reared individuals, but also in wild-caught animals, thereby offering new perspectives with respect to ecophysiological studies in insects. For studies aiming to estimate particular compounds, complementary assays can be easily added at the different steps of this method, such as the quantification of glycerol in the aqueous solvent or fructose using cold anthrone before measuring total free sugars (van Handel, 1967). Although this colourimetric assay procedure provides ecologists with general information about the whole energetic budget of an organism, it could advantageously be combined with more specialized methodologies, such as double radiolabelling, stable isotope tracking or high-performance liquid chromatography (Steppuhn & Wäckers, 2004; Wäckers *et al*., 2006), aiming to better understand the strategies of nutrient acquisition and allocation developed in insects and other arthropods, their dynamics, and their ecological and evolutionary implications.

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