N O T E

Two Adaptations of Foray's Unified Energetic Budget Measurement Protocol for Highly Chitinized Insects and Faster Glycogen Determination[1]

David Schneider,[2] Clara E. Castaños,[3] and Alex Córdoba-Aguilar[2]

Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, Ciudad Universitaria, 04510 Mexico D.F., Mexico

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The methodological study by Foray et al. (2012, Physiol. Entomol. 37: 295–302) allows for detecting and measuring energy levels in the different components of lightweight insects. The main advantage of this technique is its ease of implementation to measure sequentially total proteins, total lipids, total carbohydrates, and glycogen concentrations from a single individual. Hence, it allows a general approach of the physiologic condition of the insects, avoiding the necessity of a large number of redundant samples. Despite the ample utility of this technique, two issues have hampered its application in some cases. First, the technique of Foray et al. was originally described for lightly chitinized insects weighing no more than a few milligrams, but this could render some problems when the methodology has been applied to larger animals that grow thicker cuticles. Second, to measure glycogen concentration, Foray's unified method stipulates low-protein binding membranes filtration after an anthrone reaction with a precipitated pellet of sodium sulfate containing the glycogen. "Low-protein binding" refers to the membrane's property of binding a low quantity of proteins per unit surface area compared with other material membranes, maintaining the sample in terms of molecular compounds content as close as possible to the original. In the classic procedure, the membrane filtration was recommended because it reduces anthrone reagent turbidity (unpubl. data). However, when processing large batches, this filtration step appears to be fairly expensive and time consuming.

[1]Received 12 January 2021; accepted for publication 21 January 2021.
[2]Corresponding author (email: clara.castanos@research.uwa.edu.au).
[3]Unidad de Morfología y Función, Facultad de Estudios Superiores Iztacala, Universidad Nacional Autónoma de México, Tlalnepantla, Mexico.

Given these shortcomings, here we propose to use the following: (1) liquid nitrogen to process large and highly chitinized insects without affecting the measured concentrations of each energetic compartment, and (2) simple centrifugation rather than low-protein binding membrane filtration for glycogen concentration measurement. We reached these aims using two insects: *Zabrotes subfasciatus* (Boheman) (Coleoptera; Chrysomelidae; Bruchinae; Amblycerini) and *Tenebrio molitor* (L.) (Coleoptera; Tenebrionidae). Both insects differ in size and weight, as the mass of the former is within the range required by Foray's protocol ($<$15 mg), and the latter is greatly oversized (130–160 mg). Moreover, as with all coleopterans, both species possess heavily chitinized cuticles.

The Mexican bean weevil, *Z. subfasciatus*, attacks seeds of the common bean *Phaseolus vulgaris* L, as well as closely related Fabaceae. This pest is responsible for substantial agricultural damage on crops and stored products, mostly in the New World, as well as in Africa and Asia where the common bean is massively produced. *Tenebrio molitor* (Coleoptera, Tenebrionidae), the mealworm beetle, is distributed worldwide but native to the Mediterranean basin. Its natural habitat is forest litter or decaying material but is mostly found in granaries and stored cereal, especially if moisture has accumulated.

Wild *Z. subfasciatus* were obtained from *Phaseolus lunatus* (L.) seeds collected from the South Mexican Pacific coast near the city of Acapulco (latitude: 16°51′36.396" N, longitude: 99°52′12.864" W) and were reared for 20 generations at 27°C 12/12 light/dark. *Tenebrio molitor* larvae were obtained from four different commercial suppliers in Mexico City and the State of Mexico and were reared for 15 generations at 25°C 12/12 light/dark. Adult females of both species were selected as study specimens.

We randomly assigned 52 *Z. subfasciatus* and 52 *T. molitor* in four groups: 26 insects of each species were processed using strictly Foray's protocol (from now on ZF for *Z. subfasciatus* Foray and TF for *T. molitor* Foray), and the 26 remaining insects of each species were processed using our technique modifications (respectively, group ZN for *Z. subfasciatus* New and TN for *T. molitor* New).

Individuals from the ZN group were first crushed in liquid nitrogen with a standard ceramic mortar before being transferred into 2-ml Eppendorf tubes with 180 μl phosphate buffer (100 mM KH_2PO_4, 1 mM dithiothreitol [DTT], and 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.4). A second crushing stage was performed using a Tissue Lyser (Tissue Lyser; Qiagen, Valencia, CA) at 25 Hz for 30 s. The groups ZF and TF were processed following Foray's protocol (Foray et al. 2012), modifying the buffer quantity to maintain the corresponding Foray's concentrations for the TF group. Animals from the TN group were crushed in liquid nitrogen in a larger ceramic mortar before being transferred into 2-ml Eppendorf tubes with 1,000 μl of the same phosphate buffer and vigorously vortexed for 30 s. A second crushing stage for ZN was performed using a Tissue Lyser at 25 Hz for 30 s. In the case of *T. molitor*, because the organism was big and consequently the samples concentrated, an aliquot of 90 μl was taken from the homogenized mix and transferred into a new tube where an additional 90 μl buffer was added to reach the final volume of 180 μl required by Foray's protocol. This crushing method allows for processing large insects that would interfere with the Tissue Lyser step. Simultaneously, the vortex + aliquot + 1:2 dilution allows to extract a maximum of

material without dealing with the issues of large cuticle fragments expected from oversized hard-shelled insects.

Total proteins, total lipids, and total carbohydrates were measured following Foray et al. (2012). We decided to skip neutral lipids and fructose quantifications because we considered these subsections to be facultative options for responding to specific research questions only. However, we focused on testing whether the use of low-protein binding membranes required for glycogen optical reading could be omitted as it requires a significant investment of time for batches greater than 100 samples. Hence, we extended the glycogen quantification protocol.

After washing the sodium sulfate pellets bounded with glycogen (see p. 298 of Foray's method), 1 ml anthrone reagent was added to the pellet and then incubated 15 min at 90°C in water. The reaction was stopped on ice, as recommended, and the reacted anthrone reagent milliliters were divided into three separated 240-µl volumes. The first volume was filtered using low-protein binding membranes (polyvinylidene fluoride; d = 0.45 µm, Durapore; Millipore, Billerica, MA), the second was centrifugated 1 min at 10,000 × *g*, and the third volume was introduced directly in the microplate wells with any postprocessing. Finally, the glycogen content of all three split-samples volumes was measured using an Absorbance Reader ELx 800 spectrophotometer (BioTek, Inc., Winooski, VT) at 625 nm with glucose as the standard.

Proteins, total lipids, total carbohydrates, and glycogen concentrations were analyzed with general linear mixed models (GLMs) fit with maximum likelihood (Laplace Approximation) (Kuznetsova et al., 2017, J. Stat. Softw. 82: 1–26) assuming a gamma distribution of the residuals and with the species as random factor to test the effect of liquid nitrogen based crushing method. Secondly, a similar GLM was used to determine the effectiveness of centrifugation and membrane filtration of the anthrone reagent, with species and nitrogen-based crushing method as random factors. Third, we checked the model's validity using a simulation-based residual diagnostic tool (R package DHARMa; Hartig, 2016, Residual diagnostics for hierarchical (multi-level / mixed) regression models, R package DHARMa version 0.3.0) for hierarchical regression models before using the outputs for subsequent analysis. Finally, pair comparisons were performed with post hoc multiple comparisons of means (Tukey contrasts) to pinpoint potential flow in the new methods (R package multcomp; Bretz et al., 2011, Multiple Comparisons Using R http://www.copyright.com). We used the R environment, version 3.6.2 (R Development Core Team, 12 December 2020, http://www.r-project.org/) to generate all graphics and statistical analyses.

First, crushing the insects in liquid nitrogen did not affect the detection sensitivity of proteins, total lipids, and total carbohydrates (Table 1; Fig. 1). Moreover, note that the process permitted to measure about 30% more glycogen from *T. molitor*, but no change was observed regarding *Z. subfasciatus*. It seems likely that the cold crushing permitted an improvement of the glycogen extraction rate, but we do not discard the possibility of a protective effect of the polymer by the inactivation of glycogen degrading enzymes, given that glycogen phosphorylase is inactive at low temperatures (Graves et al., 1965, Biochem. J. 4: 290–296). Because using liquid nitrogen crushing did not provoke any significant difference in *Z. subfasciatus* glycogen determination, the most likely explanation must be purely mechanical. Given their greater mass and cuticular hardness, larger insects require a well-

Table 1. Results from the GLM post hoc multiple comparisons.

Fixed Effects	Liquid Nitrogen Pair Comparisons		Estimate	Standard Error	z Value	$P_r(>\|z\|)$
Proteins	TN	TF	0.0763	0.0705	1.081	0.701
	ZN	ZF	−0.0385	0.0719	−0.536	0.950
Lipids	TN	TF	0.0506	0.1195	0.424	0.974
	ZN	ZF	0.0305	0.1218	0.251	0.994
Carbohydrates	TN	TF	0.0126	0.1583	0.079	0.998
	ZN	ZF	0.1922	0.1613	1.191	0.632
Glycogen	TN	TF	−0.0518	0.0199	−2.600	0.045*
	ZN	ZF	−0.0055	0.0276	−0.198	0.997
Fixed Effects	**Technique Combination Comparisons**					
Glycogen Techniques	NFYS	NFNS	0.0028	0.0030	0.952	0.607
	YFYS	NFNS	0.0001	0.0029	0.024	0.997
	YFYS	NFYS	−0.0028	0.0030	−0.928	0.623

NFNS (no filter, no spin), no centrifugation + no membrane filtration; NFYS (no filter, yes spin), 1-min centrifugation at 10,000 × *g* + no membrane filtration; TF, *T. molitor* processed with original technique; TN, *T. molitor* + liquid nitrogen; YFYS (yes filter, yes spin), 1-min centrifugation at 10,000 × *g* + low- protein binding membrane filtration; ZF, *Z. subfasciatus* processed with original technique; ZN, *Z. subfasciatus* + liquid nitrogen.
*Statistically significant.

adapted grinding process to provide an adequate emulsion when fine crushed into the Tissue Lyser tubes. Surprisingly, no increased extraction rate was observed in the other energetic components (Table 1; Fig. 1), which is a fact that remains to be investigated.

Second, our results did not show any significant difference at detecting sensitivity of glycogen (Table 1; Fig. 1) neither by using membranes nor by centrifuging nor by direct reading of the anthrone reagent without any further processing. Consequently, our results indicate that reacted anthrone reagent can be directly loaded into the microplate for absorbance reading. This observation would reduce considerably the time frame of large-scale studies requiring a great number of samples or replicates, because the filtration process is the most time-consuming highly expensive step of the whole original protocol.

In conclusion, our first modification regarding the grinding of large sized and highly chitinized insects with liquid nitrogen assures a proper homogenization of the entire organism. We consider this step essential, because it subsequently allows a better extraction of glycogen for this kind of samples. With respect to the low-protein

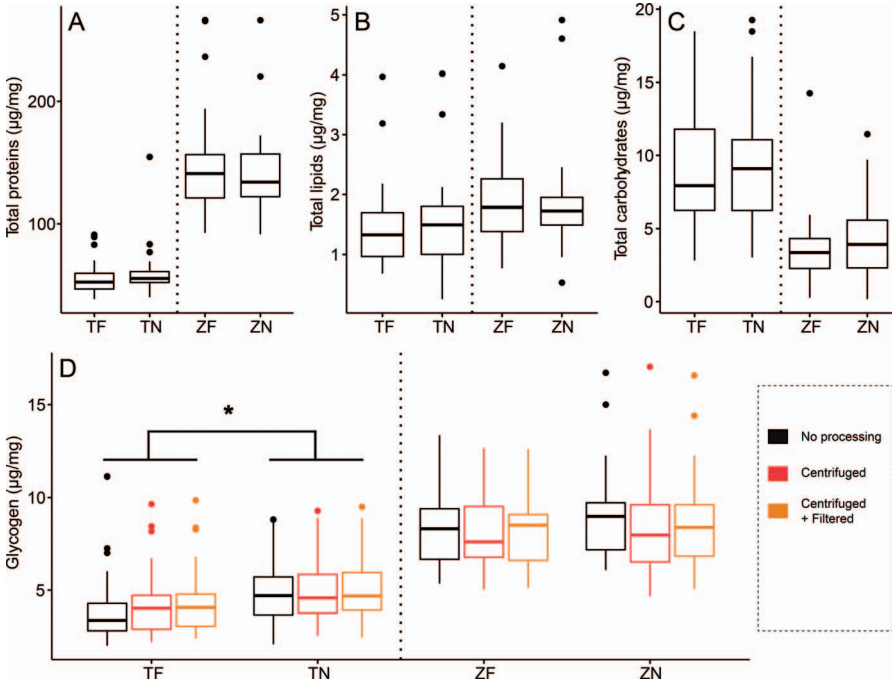

Fig. 1. Boxplots of proteins (A), total lipids (B), total sugar (C), and glycogen (D) concentrations in micrograms per milligrams of total insect mass with ZN = *Z. subfasciatus* + liquid nitrogen, ZF = *Z. subfasciatus* processed with original technique, TN = *T. molitor* + liquid nitrogen, and TF = *T. molitor* processed with original unmodified protocol.

binding membrane filtration step, this could be substituted by a simple centrifugation of the samples. Hence, this is a time- and resource-saving improvement that makes the technique easier to implement and more economically accessible.

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