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Comparing the accuracy of protein measures for arthropods

Running Headline: Accuracy of protein assays

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Abstract

Data on the protein content of arthropods can be useful for addressing a variety of ecological, behavioral, and physiological hypotheses. Yet, the most accurate method for measuring protein content (i.e., amino acid analysis) is expensive and the accuracy of less expensive measures of protein is unclear. We analyzed a diversity of arthropods to test for relationships between digestible protein content as measured by amino acid analysis and several common protein

measures: crude protein, Bradford assay, BCA assay, and Lowry assay. In the full dataset, the closest relationship to the amino acid data was found for the Lowry assay and the average of the Bradford and Lowry assays. However, one species, *Blattella germanica*, appeared to be an outlier in some analyses. When the data were analyzed without *B. germanica*, the closest relationships to the amino acid data were found for the Lowry assay. Our results suggest that not all protein measures are equal in their ability to estimate amino acid content. Some arthropod species can also contain chemicals that interfere with the accuracy of protein assays. Given that it is unclear how often interfering compounds are found in invertebrates, it may be best to conduct multiple assays when analyzing the protein content of arthropods, especially the Bradford and Lowry assays.

Keywords: Bradford Assay, BCA Assay, Lowry Assay, Crude Protein, invertebrates

Introduction

Measures of the protein content of animals are useful for examining their nutritional state as well as the nutritional content of their bodies for consumers and have been used in a variety of studies of arthropods (Wilder et al. 2013, Zaguri et al. 2021). Significant effort has been devoted to developing methods for protein quantification (Cuff et al. 2021, Zaguri et al. 2021). Unfortunately, all methods have limitations or biases and one of the most accurate methods,

amino acid analysis, is time consuming, requires specialized equipment, and is prohibitively expensive for many studies (Mariotti et al. 2008, Mæhre et al. 2018, Cuff et al. 2021, Zaguri et al. 2021). For example, a kit capable of running 2,400 microplate Lowry assays costs approximately the same as analyzing 2 samples for amino acid content. It is important to understand which of the more commonly used measures of protein, or combinations of measures, are most closely related to the more accurate amino acid analysis to allow for accurate measures of arthropod protein content until such time that amino acid analysis is more feasible for widespread use.

One of the most commonly used estimates of protein is crude protein, which is nitrogen content multiplied by 6.25 (Jones 1941, Marriotti et al. 2008, Zaguri et al. 2021). Since the development of this metric in the 19th century, it has been widely applied (Jones 1941, Marriotti et al. 2008). The crude protein measure assumes that all protein is 16% nitrogen and that all nitrogen in an animal is present in protein (Jones 1941). Yet, the nitrogen content of amino acids varies widely from 8 – 32 % (Sterner and Elser 2002). In addition, there are a variety of nitrogen-containing compounds in organisms that vary in concentration among species, including nucleic acids and chitin (Imafidon and Sosulski 1990, Sterner and Elser 2002, Elser et al. 2003). Use of the 6.25 conversion factor often results in overestimation of protein content in samples (Marriotti et al. 2008, Mæhre et al. 2018, Zaguri et al. 2021). Recognition of these criticisms of crude protein has resulted in research to quantify nitrogen-to-protein conversion factors for a variety of organisms (Jones 1941, Mariotti et al. 2008, Jonas-Levi and Martinez 2017). As likely occurs with any method estimating protein, the factors for converting nitrogen-to-protein often differ among species and can also differ within a species depending on the diet and life stage of individuals, which casts doubt on the generalizability of using such conversion factors (Jones

1941, Mariotti et al. 2008, Janssen et al. 2017, Jonas-Levi and Martinez 2017, Boulos et al. 2020, Ritvanen et al. 2020, Smets et al. 2021, Zaguri et al. 2021).

Spectrophotometric methods are another commonly used means of estimating protein content (Cuff et al. 2021, Zaguri et al. 2021). Prior to conducting spectrophotometric methods, samples are often digested in a solution such as NaOH that solubilizes soft tissue protein in a sample but not exoskeleton (Babarino and Lourenco 2005, Zaguri et al. 2021). There are a variety of spectrophotometric methods commonly used including: the Bradford assay, Lowry assay, and Bicinchoninic acid (BCA) assay (Lowry et al. 1951, Bradford 1976, Smith et al. 1985, Cuff et al. 2021, Zaguri et al. 2021). Each spectrophotometric method typically reacts most strongly with only a small subset of the amino acids present in a sample and different assays react with different subsets of amino acids (Cuff et al. 2021). Hence, variation among samples in amino acid composition can affect the accuracy of protein assays and different protein assays can result in quantitative and qualitative differences in results for both intraspecific and interspecific comparisons (Zaguri et al. 2021).

The study of protein content in arthropods is complicated due to their exoskeleton, which can vary widely as a percentange of dry mass among species (Lease and Wolf 2010). In arthropods, total protein can be broadly divided into two pools: protein in soft tissue and protein in the exoskeleton. The exoskeleton of arthropods is made of chitin, which is 7 % nitrogen, and protein, which can be up to 50 % of the weight of exoskeleton (Sterner and Elser 2002, Klowden 2007). Proteins that are bound within the chitinous matrix of the arthropod exoskeleton are: 1) not part of the metabolically-active tissue of an arthropod, and 2) indigestible to any consumer that is unable to digest chitin (Barnes et al. 2019, Wilder et al. 2019). The ability of most vertebrates to digest chitin appears limited, except for seabirds (Whitaker 1995, Cohen 1995,

Weiser et al. 1997, Barnes et al. 2019; but see Jackson et al. 1992). Chitin digestibility also appears low among invertebrates, as the majority of predatory invertebrates fail to even ingest exoskeleton, as they feed using extraoral digestion (Cohen 1995). Exoskeleton solubility also differs among the chemicals used to prepare samples for different analyses. Spectrophotometric analyses typically use chemicals that do not dissolve exoskeleton while amino acid analysis typically prepares samples using acid that dissolve exoskeleton. Crude protein also measures total nitrogen in a sample regardless of whether it is in soft tissue or exoskeleton. Hence, when analyzing protein in arthropods, it is critical to define if measures are made on total protein (i.e., protein in both soft tissues and exoskeleton) or digestible protein (i.e., protein in the soft tissue or non-exoskeleton portion of arthropods).

The goal of this study was to analyze a diverse set of terrestrial arthropods to test which measures of protein (i.e., crude protein, Bradford, BCA, Lowry, and averages of the spectrophotometric assay) were most closely related to the digestible protein content of arthropods as measured using amino acid analysis, which is considered one of the most accurate measures of protein analysis. As spectrophotometric protein assays only measure digestible protein (i.e., due to the use of NaOH or similar chemicals in sample preparation), we compared assays with digestible protein content measured through amino acid analysis (i.e., total amino acid content – amino acids in exoskeleton). In addition to ensuring the measures are quantifying the same pool of protein, digestible protein may be more relevant for studies interested in the metabolically-active tissue in an invertebrate or the nutritional quality of an invertebrate for a predator.

Methods

Data Collection

We chose 13 arthropod species for this study including one arachnid (Araneae, Araneidae, *Neoscona crucifera*, n = 5), one crustacean (Isopoda, Armadilidiidae, n = 7), and 11 different orders of insects (Blattodea, Ectobiidae, *Blattella germanica*, n = 7; Coleoptera, Scarabeidae, *Cotinus nitida*, n = 2; Diptera, Tabanidae, n = 4; Ephemeroptera, Ephemeridae, *Hexagenia* sp., n = 5; Hemiptera, Coreidae, *Anasa tristis*, n = 6; Hymenoptera, Vespidae, n = 7; Lepidoptera, Nymphalidae, *Asterocampa celtis*, n = 4; Mantodea, Mantidae, *Stagmomantis carolina*, n = 2; Odonata, Libellulidae, *Plathemis lydia*, n = 2; Orthoptera, Acrididae, *Syrbula admiralis*, n = 2; Phasmatodea, Diapheromeridae, n = 2). For each species, we created two pooled samples of individuals: one for measuring nutrients in exoskeleton and one for measuring nutrients in the whole body. We chose species to represent a diversity of Orders and body forms. We used one species per Order and identified them to Family, or Species for more common taxa. Arthropods were collected around Stillwater, Oklahoma USA.

Samples were first dried at 60 ºC for 24 hours and then analyzed for lipid content using the gravimetric method with chloroform (Cuff et al. 2021). Lipid was removed from samples to prevent it from interacting with NaOH during the protein extraction. One to four individuals of each species were then used for determination of exoskeleton content, which was measured as percent of dry mass. Greater numbers of individuals were used for smaller bodied species. Briefly, these samples were weighed, the exoskeleton was broken open to allow the NaOH to enter the body and head, soaked in 0.1 M NaOH, and the remaining exoskeleton was weighed (Lease and Wolf 2010, Cuff et al. 2021). The other individuals of each species were ground into a powder and the powder was divided for elemental analysis, protein assays, or to be sent away for amino acid analysis. Samples for elemental analysis were packaged in tin capsules containing

1.5 – 3 mg of dried and ground tissue and analyzed in a CHN analyzer using the Dumas method. Crude protein was calculated as nitrogen content multiplied by 6.25 (Jones 1941). For protein assays, we dissolved 5 – 9 mg of powder in 1 mL of 0.1 M NaOH and placed samples in a sonicator at 80 ^{o}C for 30 minutes. After centrifuging at 13,000 rpm for 10 minutes, the supernatant was analyzed for protein content using Bradford (i.e., Coomassie Plus Assay Kit, Thermo Scientific), BCA (Pierce BCA Protein Assay Kit, Thermo Scientific), and Lowry (Pierce Modified Lowry Protein Assay Kit, Thermo Scientific) protein assays according to the manufacturer instructions for use of the assays with 96 well microplates. For each protein assay, we ran three technical replicates of each sample. We used pre-diluted bovine gamma globulin standards (Thermo Scientific) in microplate assays with a standard curve including 8 concentrations from 0 to 2 mg/mL of protein.

Amino acid analysis was used to measure the protein content of both the whole body (i.e., containing soft tissue and exoskeleton) and just the exoskeleton of each of the 13 arthropods. Amino acid analysis was conducted by AAA Service Lab (Damascus, OR, USA) using a Hitachi L8900 Amino Acid Analyzer. This analysis quantified concentrations of the 16 most common amino acids. This analysis did not quantify cysteine and tryptophan, which for arthropods are typically the least abundant amino acids and each comprise 1.5 percent or less of total amino acid content (Boulos et al. 2020, Ritvanen et al. 2020, Smets et al. 2021). Hence, we assumed that the results of amino acid analysis were a close approximation of the protein content of arthropod samples.

The acid hydrolysis used to prepare samples for amino acid analysis digests exoskeleton and releases the amino acids bound in the chitin matrix of the exoskeleton. However, the chemicals typically used to solubilize protein samples (i.e., NaOH) do not digest exoskeleton,

which means that the protein assays should be measuring only those proteins in the soft tissue and not the exoskeleton of arthropods. Hence, we calculated digestible (i.e., soft tissue) amino acid content of samples by measuring the amino acid content of whole arthropods, amino acid content of only exoskeleton, and the proportion exoskeleton of each arthropod (i.e., digestible AA = whole arthropod AA – exoskeleton AA * proportion exoskeleton).

Statistical Analysis

All analyses were conducted in R Studio (2022.02.3, Build 492). Linear regression analyses were conducted to test the relationship between soft tissue amino acid content of samples (predictor variable) and protein measure values (response variable) using individual protein measures and averages of two or three spectrophotometric assays. We did not include crude protein in the averages of assays because it is very different in the way that it estimates protein relative to the spectrophotometric assays. For the spectrophotometric assays, once protein is extracted it is relatively easy to conduct multiple assays. Hence, we were interested in testing if one or the averages of multiple spectrophotometric assays were more closely related to amino acid content.

Four measures were used to judge which estimates of protein were the closest to the amino acid values, with the best measure(s) being that which satisfied all criteria (Table 1). First, the relationship between a protein measure and amino acid content would have a slope greater than zero, which indicates that there is a significant linear relationship between the variables. Second, the relationship would have a slope that was not significantly different from 1, which would indicate that the protein measure had a direct correspondence with amino acid content. This was assessed using an offset in linear regression. Third, the relationship would have a y-

intercept that was not significantly different from 0. Finally, the delta AIC for the relationship between amino acids and the protein measure should have a value less than 2. The AIC value measures the relative fit of different models for the same response variable. To calculate AIC values, separate regressions had to be done using amino acid content as the response variable and the protein measures as the predictor. Delta AIC measures the difference in AIC values between the best fit model and other models. Any models with delta AIC less than 2 were considered to have a strong fit to the data. We used the aictab function in R to calculate delta AIC values for all models.

Finally, we conducted a Friedman test to compare the median values among amino acid analysis and the individual protein measures for the dataset excluding *B. germanica*. A Wilcoxon signed-rank test with Bonferroni-adjusted p-values was used to examine pairwise posthoc differences.

Results

Full Dataset

For the full dataset, the slopes of the relationships between protein measures and amino acid content ranged from -0.14 – 1.25, the y-intercepts from 1.35 – 41.05, and the R^2 from 0.004 – 0.66. There were only three assays that had statistically significant relationships with amino acid content as demonstrated by slopes > 0: Bradford, Lowry, and the average of the Bradford and Lowry assays (Table 1, Figures 1 and 2). All three of these assays also had slopes that were not significantly different from 1 and y-intercepts that were not significantly different from 0. Hence, all three of these assays had 1:1 relationships with amino acid content. Two of these

assays, Lowry and the average of the Bradford and Lowry, had delta AIC less than 2, indicating that they also had the highest fit for the data.

Dataset excluding B. germanica

In several of the analyses, *B. germanica* appeared to be an outlier. For example, the crude protein content of *B. germanica* was 95.1 % while the nitrogen content of the next highest arthropod *Plathemis lydia* was 78.3 % and the average of all arthropods excluding *B. germanica* was 67.4 $\pm$ 3.9 (mean $\pm$ 1 SE). Also, the BCA analysis of *B. germanica* reported a protein content of 102 % while the BCA protein content of the next highest arthropod *Hexagenia* sp. was 34.7 % and the average of all arthropods excluding *B. germanica* was 24.9 $\pm$ 1.9 %. Hence, we also analyzed the data excluding *B. germanica*.

For the dataset excluding *B. germanica*, the slopes of the relationships between protein measures and amino acid content ranged from 0.59 – 1.27, the y-intercepts from -1.33 – 24.5, and the R^2 from 0.49 – 0.90. There were significant positive relationships (i.e., slope > 0) between soft tissue amino acid content and all measures of protein separately and for the averages of the spectrophotometric assays (Table 1, Figures 1 and 2). All except two of these relationships also had a slope that was not significantly different from 1. The BCA and average of the BCA and Lowry assays had slopes that were less than 1. Five of the remaining assays also had y-intercepts that were not significantly different from 0: Bradford, Lowry, the average of BCA and Bradford, the average of Bradford and Lowry, and the average of all spectrophotometric assays. Satisfying these first three criteria indicated that these five assays were not significantly different from 1:1 relationships with amino acid content. Comparison of delta AIC showed that the Lowry assay had the highest fit for the data out of these five assays.

Finally, there were significant differences between median protein values of the amino acid analysis and the protein measures (Figure 3; df = 4, $X^2 = 42.1$, $p < 0.0001$). The median Bradford Assay was not significantly different from that of amino acid analysis; although, variation in the Bradford Assay was high. The BCA assay was significantly lower and the crude protein measure was significantly higher than amino acid analysis. The Lowry Assay was also significantly lower than amino acid analysis; although, the difference in median values between amino acid analysis (41.4) and the Lowry Assay (38.2) was low.

Discussion

The results of this study suggest that, for arthropods, there are differences in the relationships between common protein measures and amino acid content, which is considered to be one of the most accurate measures of protein content. Some assays produced lower protein values (e.g., BCA) while others produced higher protein values (e.g., crude protein) compare to amino acid analysis. Differences among the spectrophotometric assays are likely because each assay interacts with only a subset of amino acids in samples (Bradford 1976, Jones et al. 1989, Cuff et al. 2021). For the full dataset, the Lowry assay and the average of the Bradford and Lowry assays were closer to values for amino acid analysis than the other assays. For the data excluding *B. germanica*, the Lowry assay was closer to values for amino acid analysis than the other assays (Table 1). While the Lowry assay was close to amino acid values in both datasets, it may be useful to use multiple assays to analyze protein samples in the future, to ensure that the results are consistent among assays (e.g., Zaguri et al. 2021).

It is important to note that we used bovine gamma globulin as a standard protein for this study. Bovine serum albumin is another very common protein standard. The choice of protein

standard will affect the estimate of protein content in unknown samples with the effect differing by assay type. Compared to BSA, BGG results in higher protein estimates for the Bradford assay, lower protein estimates for the BCA assay, and lower protein estimates for the Lowry assay (Zaguri et al. 2021; *see also* manufacturer's instructions for each assay from Thermo Scientific).

Another important result from this study is that there can be compounds in organisms that interfere with protein assays and significantly affect their accuracy (Mæhre et al. 2018). In this study, it was clear that *B. germanica* had compounds in their body that interfered with the BCA assay and also possibly the Lowry assay (Figure 1). It was also an outlier for the crude protein calculations. Some cockroaches, like *B. germanica*, can accumulate significant amounts of uric acid in their bodies as a nitrogen storage reserve (Valovage and Brooks 1979, Cochran 1985). Uric acid can affect the accuracy of the crude protein measure and interfere with the BCA protein assay and potentially other spectrophotometric protein assays. Arthropods are diverse in their biochemistry (Klowden 2007), including in chemical defenses, and each protein measure has a range of chemicals that can potentially interfere with protein estimates. Fortunately, differences in the chemistry of the protein assays make it possible for chemicals to interfere with one assay but less with others (Zaguri et al. 2021). Hence, it may be useful to conduct multiple protein assays on the same sample to ensure that there are not large differences between them. Once protein has been solubilized, running additional protein assays is relatively quick and easy, especially if using the procedures for running samples on 96-well microplates.

Another potential solution to the problem of interfering compounds is to use a protein purification method, such as those that use TCA for protein precipitation (e.g., Barbarino and Lourenco 2005). Although, whether or not these protein purification protocols remove all

interfering compounds is unclear. It is also unclear if the process of protein purification affects the accuracy of protein assays. Protein purification could remove free amino acids, which may or may not be important to include in measures of the protein or amino acid content of samples. In addition, some protocols suggest that details of certain steps in protein purification can affect protein yield (Fic et al. 2010, Niu et al. 2018). Further work on the effects of protein purification on interfering compounds and the accuracy of protein assays in diverse species would be valuable.

Our data support the findings of previous studies that calculations of crude protein using the 6.25 factor overestimate the protein content of arthropods, in terms of what is measured during protein assays or is digestible to those consumers with limited ability to digest chitin (Marriotti et al. 2008, Mæhre et al. 2018, Zaguri et al. 2021). Interestingly, in the dataset excluding *B. germanica*, the slope of the relationship for crude protein was close to one and the R^2 was reasonable. However, the y-intercept for the relationship was 24.5, which indicates that crude protein consistently overestimated protein content by this amount, on average. Interestingly, the average exoskeleton content of arthropods was 27.6 % and the amino acid content of exoskeleton (40.6 $\pm$ 4.8 %) was not much lower than that of the whole arthropods (50.4 $\pm$ 2.8 %). In addition, the nitrogen content of chitin, which is about 7 %, would further increase the estimated crude protein content of exoskeleton. Crude protein includes all amino acids (i.e., both in digestible soft tissue and indigestible exoskeleton) and all nitrogen-containing compounds (e.g., chitin, nucleic acids) in the calculation of protein content and the amino acids in exoskeleton and N in chitin appear to be important factors resulting in overestimation of protein content in arthropods by the crude protein assay.

In conclusion, protein assays offer an easy and affordable method for estimating the nutritional content of arthropods. However, no single protein assay appears ideal for estimating the protein content of invertebrates and there are tradeoffs associated with different assays (Cuff et al. 2021, Zaguri et al. 2021). Data suggest that for some species, the Lowry assay may be a reasonable approximation of protein content. However, it may be better to conduct multiple protein assays on the same samples to confirm that the results of a study are robust and consistent across assays. The average value for the Bradford and Lowry assays was closely associated with digestible amino acid content in the full dataset (Table 1). It may also be worthwhile to conduct pilot studies comparing amino acid content with the various protein assays for model organisms or those species on which extensive research will be conducted to test if a single assay works well for particular species. Such studies should include individuals raised on different diets or under diverse conditions (Smets et al. 2021, Zaguri et al. 2021). Ideally, using amino acid analysis to calculate digestible protein content will be more widely adopted as the cost for this method becomes lower in the future. However, until that time, it is important to validate the assays that are used as approximations of protein content and to use those methods that are best supported by data.

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Declarations of interest: none

Author Contributions: SMW and CLB conceived of the ideas, designed the methodology, collected the data, contributed critically to the drafts and gave final approval for publication. SMW analyzed the data and led the writing of the manuscript.

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Table 1. Results of linear regressions and analyses comparing the soft tissue amino acid content of samples with individual or averaged protein assays. Values shaded in gray meet the criteria for a relationship with amino acid content for each analysis. Those assay or combinations of assays that meet all four criteria represent the closest relationship with amino acid content.

Protein Assay	Slope	Intercept	R2	p Slope > 0	p Slope = 1	p Intercept	delta AIC
Full Dataset							
Crude	0.73	41.05	0.24	0.09	0.51	0.03	10.7
Bradford	1.25	1.35	0.51	0.006	0.51	0.93	4.9
Lowry	0.77	6.89	0.62	0.001	0.24	0.36	1.6
BCA	-0.14	36.25	0.004	0.84	0.12	0.21	14.2
Ave of BCA and Lowry	0.31	21.68	0.05	0.47	0.13	0.22	13.6
Ave of BCA and Bradford	0.56	18.72	0.15	0.19	0.29	0.27	12.1
Ave of Bradford and Lowry	1.01	4.12	0.66	< 0.001	0.95	0.65	0.0
Ave of Bradford, BCA and Lowry	0.63	14.81	0.26	0.07	0.27	0.27	10.3
Excluding *Blattella germanica*							
Crude	1.08	24.50	0.63	0.002	0.77	0.046	16.0
Bradford	1.27	0.52	0.49	0.01	0.52	0.98	19.7
Lowry	0.95	-1.33	0.90	< 0.001	0.59	0.74	0.0
BCA	0.59	1.48	0.75	< 0.001	0.003	0.74	11.0
Ave of BCA and Lowry	0.77	0.16	0.86	< 0.001	0.04	0.97	4.0
Ave of BCA and Bradford	0.93	0.93	0.62	0.003	0.78	0.92	16.4
Ave of Bradford and Lowry	1.11	-0.42	0.72	< 0.001	0.63	0.96	12.7
Ave of Bradford, BCA and Lowry	0.94	0.20	0.75	< 0.001	0.71	0.98	11.0

Figure Legends

Figure 1. The relationships between percent protein estimated using individual assays of protein and percent digestible protein calculated using the results of amino acid analysis for datasets that either excluded (solid points and dark solid line) or included (all points and

dashed line) *Blattella germanica*. A reference 1:1 line is included as a thinner diagonal line. The data for *B. germanica* are in open circles.

Figure 2. The relationships between percent protein estimated using the averages of pairs or all spectrophotometric assays of protein and percent digestible protein calculated using the results of amino acid analysis for datasets that either excluded (solid points and dark solid line) or included (all points and dashed line) *Blattella germanica*. A reference 1:1 line is included as a thinner diagonal line. The data for *B. germanica* are in open circles.

Figure 3. Boxplot comparing the protein values calculated for amino acid analysis and the four protein measures for the dataset excluding *Blattella germanica*. Bars with different letters were significantly different from each other in posthoc analysis.

Figure 1.

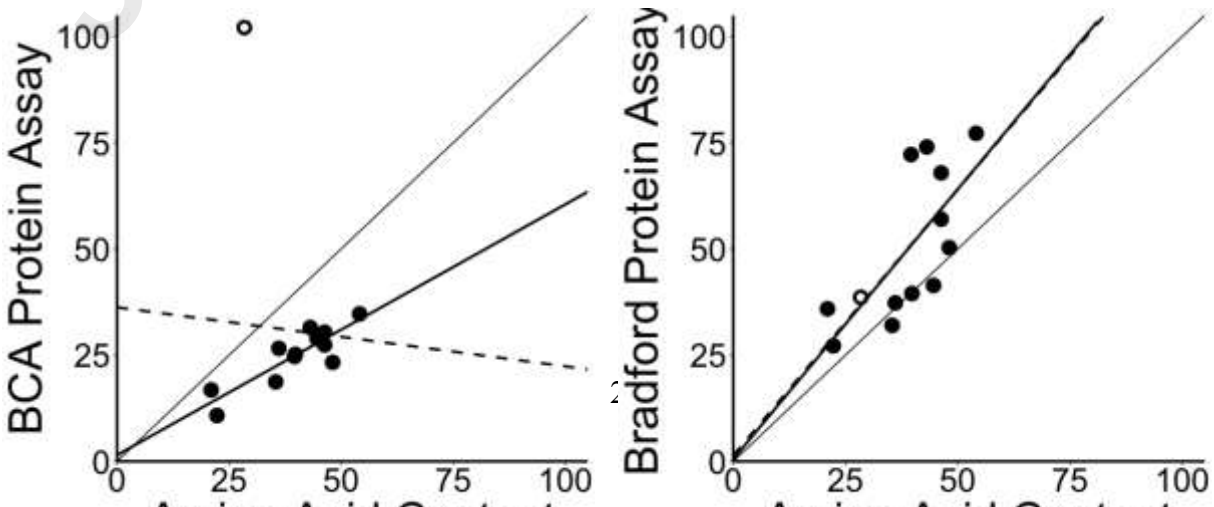
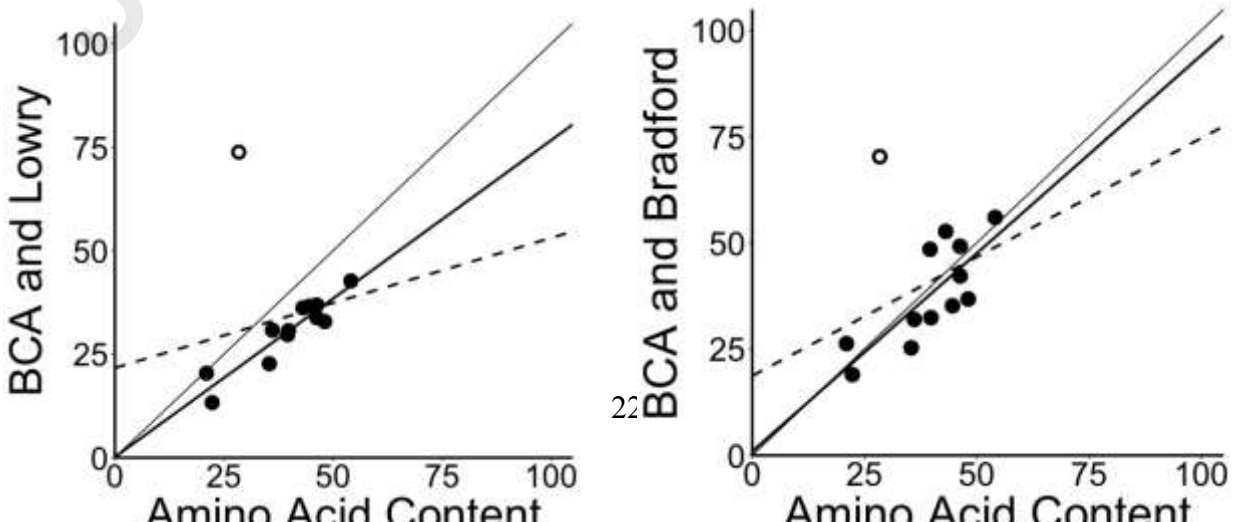


Figure 2.



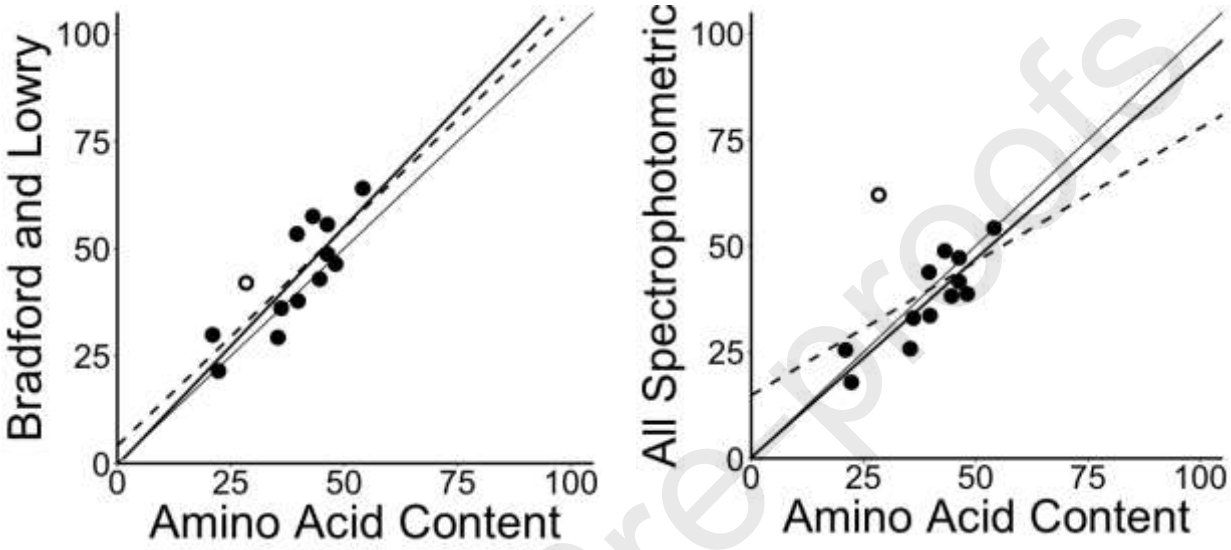
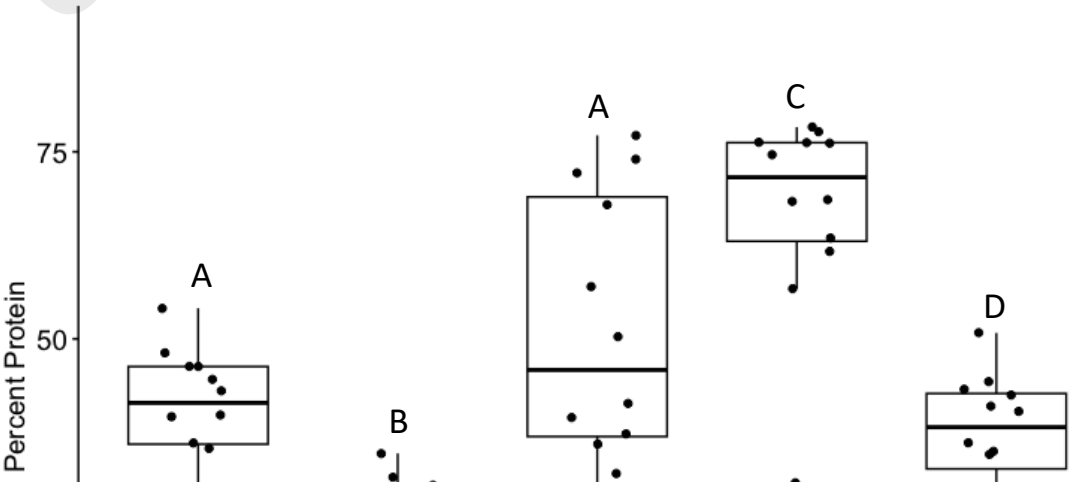


Figure 3.



Highlights

- Amino acid analysis is an accurate measure of protein but expensive
- Interfering compounds can affect the accuracy of protein estimates
- Crude protein overestimates digestible protein in arthropods
- The Lowry assay is closely related to amino acid content
- Average of Bradford and Lowry was also closely related to amino acid content

CReDiT Author Statement

Shawn Wilder: Conceptualization, Methodology, Formal analysis, Investigation, Resources, Data curation, Writing-Original Draft, Writing-Review and Editing, Visualization, Funding acquisition
Cody Barnes: Conceptualization, Methodology, Investigation, Writing-Review and Editing

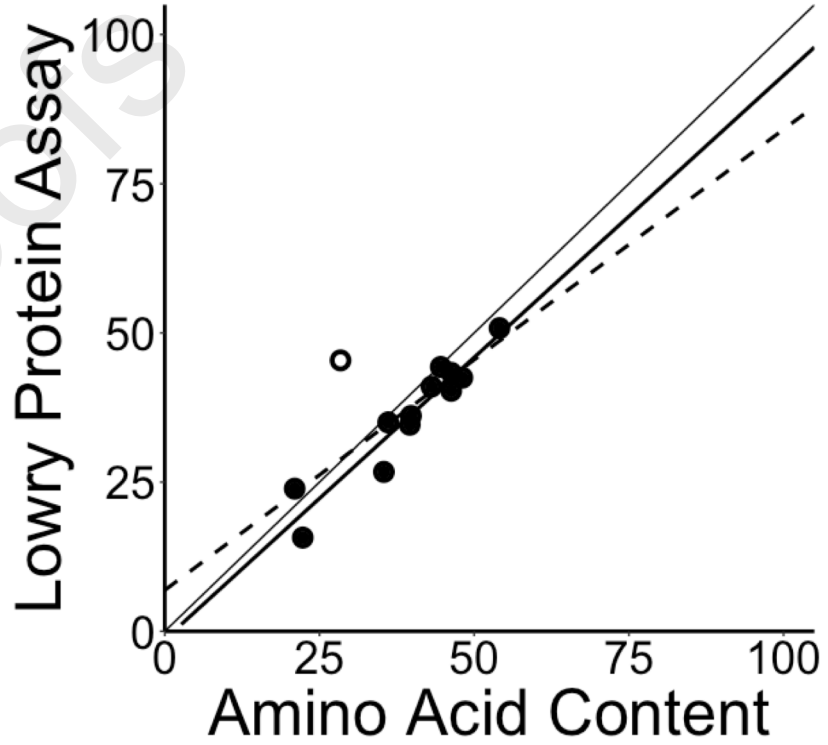
How best to measure arthropod protein?

Amino Acid Analysis

- Expensive but accurate
- How does it compare to assays?

Crude Protein (Nitrogen x 6.25)

- Overestimates protein
- Measures all N and not all N is protein



Spectrophotometric

- Susceptible to interfering compounds
- Lowry or the average of Bradford and Lowry were most closely related to amino acid content