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RESEARCH ARTICLE

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Protein quantification in ecological studies: A literature review and empirical comparisons of standard methodologies

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Abstract

1. Protein quantification is a routine procedure in ecological studies despite the inherent limitations of well-acknowledged protein determination methods which have been largely overlooked by ecologists. Thus, we want to bridge this knowledge gap, in hopes of improving the way ecologists quantify proteins and interpret findings.
2. We surveyed the ecological literature to determine how and why ecologists quantify proteins. To determine whether different quantification methods produce comparable results across taxa, and between populations of a single species, we estimated the protein content of eight phylogenetically diverse taxa, and of desert isopods fed different diets, using various derived protocols of the 'crude protein', Bradford and bicinchoninic acid approach (BCA) methods.
3. We found that ecologists use many protein quantification procedures, often without reporting the crucial information needed to evaluate and repeat their methods. Our empirical work demonstrated that the three quantification methods examined, and their derived protocols, resulted in highly divergent protein estimations that were inconsistent in rank across taxa, preventing conversion between methods. We also found that different quantification methods yielded different answers to whether isopod protein content is affected by diet.
4. We conclude that commonly used quantification techniques yield distinct protein estimations with varying precision, and no single method is likely to be more accurate than another across taxa which may lead to inconsistent results across taxa and between conspecifics. Inaccurate protein quantification may explain the observed mismatch between organismal N and protein that has plagued some recent studies and that contradicts the principles of ecological stoichiometry. We recommend using a single BCA protocol to reduce inconsistencies across studies, until the promising amino acid analysis becomes more affordable, accurate and accessible to ecologists. Until then, ecologists should consider the abovementioned drawbacks of protein quantification methods and interpret their results accordingly.

KEYWORDS
bicinchoninic acid approach, bradford, crude protein, ecological stoichiometry, nitrogen-to-protein factor, nutritional ecology, protein quantification

1 | INTRODUCTION

Protein quantification is routinely done in ecological research (Steuer et al., 2014; Tigreros, 2013; Zhang et al., 2016). Ecologists quantify proteins to understand the nutritional values of food resources (Cruz-Rivera & Hay, 2000; Felton et al., 2009), to reveal organismal responses to different biotic and abiotic conditions (Bielski et al., 2018; Hernandez-Cumplido et al., 2016), and to standardize enzyme activity or ensure equal loading of electrophoresis gels (Serafin et al., 2017). In recent years, protein quantification methods accessible to ecologists have increased, bringing new opportunities, but also potential risks if used or reported erroneously. Our overarching goal was to reveal these potential risks and improve how ecologists quantify proteins and interpret their findings.

The diverse protein quantification methods can be divided to several principle approaches. One common indirect approach is based on the sample's nitrogen content, commonly determined by the Dumas combustion or the Kjeldahl methods. This approach is more precise than alternative methods, but requires a presumed amino acid composition and non-protein nitrogenous compounds content (e.g. nucleic acids, amines, chitin, etc.) to convert the measured N content to crude protein (CP) estimation (Mariotti et al., 2008). Traditionally, CP is estimated by multiplying the nitrogen content by a nitrogen-to-protein conversion factor of 6.25 (Moore et al., 2010). Yet, it is widely accepted that any single conversion factor cannot provide accurate CP assessments across different tissues or organisms (Mariotti et al., 2008) since (a) nitrogen content varies substantially between proteins (13.4%–19.3%; Jones, 1941) depending on their amino acid composition, and (b) the non-protein nitrogenous substance fraction is highly variable and depends on the sample type and the extraction and purification processes (Mariotti et al., 2008).

Other popular indirect ways to quantify protein in biological samples are spectrophotometric approaches, including near and far UV-absorption methods (Gill & von Hippel, 1989; Scopes, 1974), and colorimetric approaches, including the Coomassie brilliant blue (i.e. Bradford; Bradford, 1976), Biuret assay, Lowry's method (Lowry et al., 1951), and the bicinchoninic acid approach (BCA; Smith et al., 1985). The spectrophotometric and colorimetric approaches require that the protein first be extracted. Extraction protocols can dramatically affect the total protein estimation of a sample (Barbarino & Lourenço, 2005) and deserve a thorough separate examination that is beyond the scope of this paper. The two quantification approaches do not require pre-calculated conversion factors, but are based on specific protein properties (Olson & Markwell, 2007). For instance, high relative concentrations of tyrosine and tryptophan leads to protein overestimations in the UV 280-nm absorbance method (Simonian & Smith, 2006), while high relative concentrations of arginine leads to protein underestimations in the Bradford method (Compton & Jones, 1985). These approaches are also prone to interference from other compounds existing in ecological samples. Nucleic acids can influence protein quantification by UV absorption (Simonian & Smith, 2006), while the Lowry method is sensitive to many substances, including most phenols, and uric acid (Peterson, 1979). Additionally, spectrophotometric and colorimetric methods have different sensitivities to chemicals used in various protein extraction methods as well as to free amino acids (Compton & Jones, 1985; Walker, 2009).

A more direct method to quantify total protein content is the amino acid analysis (AAA). Briefly, peptide bonds are hydrolysed and the liberated amino acids are then separated, detected and quantified (Rutherfurd & Gilani, 2009). In theory, the resulted estimations are not affected by the amino acid composition, non-protein nitrogenic compounds and interfering substances. Yet, it is very laborious and complicated to achieve such accuracy (Rutherfurd & Gilani, 2009). Moreover, AAA is expected to suffer from inaccuracy due to degradation of amino acid or differential protein resistance during the initial hydrolysis stage (Rutherfurd & Gilani, 2009). Likewise, the inclusion of free amino acids, which may reach more than 10% of the protein content, can also bias the total protein estimation (Helland et al., 2003; Moore et al., 2010; Smith, 2017). As of today, AAA is expensive and not accessible as a routine protein determination method to most ecologists.

As clearly demonstrated by our brief overview, different protein quantification methods are expected to produce different protein estimations with varying precisions (Olson & Markwell, 2007). This problem is well-known in various fields such as biochemistry, pharmacology and nutrition (Knight & Chambers, 2003; Moore et al., 2010). However, we found no similar discussion in the ecological literature. This is surprising given the potentially important empirical and conceptual implications of these caveats for ecological research. To bridge this knowledge gap, we conducted an extensive literature review and two empirical studies asking: (a) how and why do ecologists quantify proteins, (b) are common protein quantification methods comparable across taxa and (c) do protein quantification methods produce qualitatively similar results in comparisons of conspecifics. In addition, we discuss the possible implications of these methodological drawbacks to ecological theory and recommend how (and how not) to quantify and report total protein in ecology.

2 | LITERATURE REVIEW

2.1 | Materials and methods

To determine how and for what purposes ecologists quantify proteins, we surveyed the literature using the Web of Science (WoS)

Core Collection database with the following search terms: 'protein quantification' OR 'protein content' OR 'total protein'. We restricted our search to between 1 January 2009 and 24 February 2019 (when the search was conducted), to the 160 'Ecology' journals defined by WoS. This primary search yielded 226 papers (see Appendix S1), of which only 177 quantified proteins. From each paper, we extracted information regarding the organisms used, the reason for protein determination, and various aspects of the quantification method (e.g. the conversion factor for CP, wavelength, protein standards and kits used for colorimetric methods; Appendix S2). When other sources were cited instead of providing detailed protocols, we used the cited information to complete the missing details. We could not complete the missing data when the cited sources included several protocols [e.g. Association of Official Analytical Chemists—Official Methods of Analysis (AOAC)] without mentioning the specific one or cited the original Bradford and BCA method papers, because a preliminary search revealed that studies citing these two sources often deviated from the original protocol. When studies used more than one quantification method, we extracted each method's relevant information and treated them as separate studies.

In addition to searching for studies that quantified protein, we explored the various and detailed protocols used for three popular methods: CP, Bradford and BCA. BCA is commonly used in biochemistry due to its high stability under alkali conditions, and tolerance to interference compounds (Walker, 2009). We arbitrarily chose 79 papers per method to make sure we obtained enough papers to thoroughly explore the protocols' intra-method variations. For this secondary search, we first used the terms 'crude protein' OR 'Bradford assay' OR 'BCA'. This search yielded an additional 119, 4 and 2 papers for CP, Bradford and BCA, respectively. Second, we conducted a 'Citing Articles' search for both Bradford (1976) and Smith et al. (1985), using the same restrictions as our primary search. We found 822 and 35 papers that used Bradford and BCA methods, respectively. To meet our quota of 79 papers, we extracted data from the 13 most recent Bradford papers and all 35 BCA papers. Third, we completed our BCA paper quota by conducting a complementary literature search using Google Scholar and the search term 'BCA protein' within a randomly selected group of Ecology journals (30% of the list of journals classified as 'Ecology' by WoS). We added the 34 most recent papers of this search to our dataset.

2.2 | Results

Of the total 492 papers examined, only 410 quantified proteins in an ecological context, and four papers used two different quantification methods (overall 414 studies; for the full list, see Appendix S3, Tables S1–S3). Of the 180 studies from the primary search, 9% quantified proteins to standardize other metrics, 9% quantified proteins to validate their methodology, and 95% quantified the protein content of their study organism (the sum exceeds 100% because several papers quantified proteins for multiple purposes). Considering all the 414 studies, the percentage of studies seeking methodological validation or actual protein content was slightly lower (6%, and 87% respectively), and higher for standardization purposes (16%). These differences may stem from the tendency of using BCA for standardization. From the studies that quantified total protein content, 91% from both the initial 180 and total 414 studies, explore how different rearing conditions affect the nutrient content of conspecifics. Ecologists were also interested in understanding their organisms' absolute protein content to derive their nutritional value in a food-web context (26% and 28% for the primary search and total number of studies, respectively).

Our primary literature survey revealed 19 distinct protein quantification methods. The most common methods were the Bradford method (34%), techniques for nitrogen quantification and CP estimation (26%), and the Lowry assay (14%). BCA (4.4%) and the Biuret assay (5%) were used less frequently. The near infrared spectroscopy (NIRS; 1.7%) and AAA (0.5%) were rarely used. Most studies aimed to quantify the protein content of animal or plant samples (47% and 38%, respectively). Of the studies that quantified protein in plants, the majority used CP or the Bradford method (37% each). Studies that quantified proteins in animals used more diverse methodologies. This bias may reflect cultural differences in methodology or reporting styles between disciplines.

Only 78% of the studies that used CP reported the detailed N-quantification method. Of these, 56% used the Kjeldahl method and 44% used combustion-based methods. Only 53% of the studies that reported CP provided the conversion factor used. Of these, 90% used the popular 6.25 nitrogen-to-protein conversion factor. The others used conversion factors ranging from 4.78 to 6.08.

Only 61% of the Bradford method studies reported the type of protein standard used for calibration. Of these, 94% used bovine serum albumin (BSA; Table 1). Only 8% of the Bradford studies reported the time between reagent addition and absorbance measurement (incubation time), which ranged from 5 to 30 min, and only 5% reported the incubation temperature that ranged from 20 to 37°C. Only 15 studies specified the calibration curve used, of which 14 reported a 'standard curve'. This vague term possibly means a linear curve (Y. Eder, J. Wehr, personal communication). One-fourth of the studies used a commercial kit or reagent to conduct their Bradford assay.

Only 56% of the BCA-method studies reported the type of protein standard used for calibration. Of these studies, 95% used BSA (Table 1). Studies using BCA rarely reported the calibration curve used. Thirteen studies reported a 'standard curve', and one study used a quadratic curve (Movellan et al., 2012). The incubation time when reported (11%), ranged from 30 to 1,440 min, and those reporting incubation temperature (11%) ranged from 20 to 37°C. Most BCA studies (78%) used a commercial kit or reagent, with a clear preference (76%) for the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific).

TABLE 1 Methodological details reported for the studies using Bradford or BCA methods. Data present the number of studies and percentages in parentheses (out of 79 total studies per method) using each methodological approach

	Standard protein			Calibration curve			Wavelength (nm)			Incubation time (minutes)			Incubation temperature (°C)			Commercial kit or reagent		
Bradford	BSA	45	(57%)	Linear	1	(1.3%)	595	34	(43%)	5	3	(3.83%)	RT	1	(1.3%)	Bio-Rad	11	(16.4%)
	IgG	2	(2.5%)	Cubic	0	(0%)	590	1	(1.3%)	10	2	(2.5%)	25	1	(1.3%)	Sigma-Aldrich	1	(1.5%)
	Other	1	(1.3%)	Quadratic	0	(0%)	595:465	1	(1.3%)	30	1	(1.3%)	30	1	(1.3%)	Pierce/ Thermo	0	(0%)
	NA	31	(39.2%)	Standard	14	(17.7%)	565	1	(1.3%)	NA	73	(92.4%)	37	1	(1.3%)	Other	3	(4.5%)
				Other	0	(0%)	NA	42	(53.2%)				NA	75	(94.9%)	NA	52	(77.6%)
				NA	64	(81%)												
BCA	BSA	42	(53.2%)	Linear	0	(0%)	562	23	(29.1%)	30	4	(5.1%)	RT	1	(1.3%)	Bio-Rad	0	(0%)
	IgG	0	(0%)	Cubic	1	(1.3%)	540	1	(1.3%)	15	1	(1.3%)	25	1	(1.3%)	Sigma-Aldrich	9	(11.4%)
	Other	2	(2.5%)	Quadratic	0	(0%)	550	2	(2.5%)	20	1	(1.3%)	37	4	(5.1%)	Pierce/ Thermo	47	(59.5%)
	NA	35	(44.3%)	Standard	13	(16.5%)	750	1	(1.3%)	60	1	(1.3%)	45	1	(1.3%)	Other	6	(7.6%)
				Other	0	(0%)	NA	52	(65.8%)	720	1	(1.3%)	60	2	(2.5%)	NA	17	(21.5%)
				NA	65	(82.3%)				1,440	1	(1.3%)	NA	70	(88.6%)			
										NA	70	(88.6%)						

3 | EMPIRICAL STUDY

3.1 | Materials and methods

3.1.1 | Experimental design

We conducted two separate experiments. The first explored whether different protein quantification techniques yield similar protein content estimations across taxa. We used the three popular methods: CP, Bradford and BCA, to estimate the protein content of sea anemones *Nematostella vectensis*, desert snails *Trochoidea simulata*, spiders *Holocnemus pluchei*, two crustacean species (freshwater zooplankton *Daphnia magna* and desert isopod *Hemilepistus reaumuri*) and three insect species (locust *Schistocerca gregaria*, honey bee *Apis mellifera* and fruit fly *Drosophila melanogaster*). We pooled together 20–80 individuals of each species, depending on their biomass, to reach a sufficient sample quantity. All organisms were freeze-dried for 48 hr, manually ground with a pestle and mortar, and then powdered and homogenized using a mixer mill (MM 400; Retsch). For each of the two principal colorimetric methods (Bradford and BCA), we used varied protocols that included two protein standards (BSA and BGG) and three or four calibration methods (for BCA and Bradford, respectively). We tested how these 14 quantification techniques and different incubation times affect protein estimations across taxa. Each quantification technique's precision (overall 15 techniques including the CP) was evaluated.

The second experiment explored whether different quantification techniques produce qualitatively similar results when comparing the protein content of conspecifics. We experimentally fed desert isopods with four different diets: (a) plant litter, (b) plant litter and soil crust, (c) plant litter supplemented with calcium and (d) plant litter supplemented with calcium and phosphorus. We quantified protein content in 12 randomly chosen isopods from each treatment group, using the 15 techniques used in the first experiment. We prepared the samples as in the first experiment, but rather than mixing all individuals, we analysed each of the 48 isopods separately, using all the 15 techniques. This approach enabled assessing intraspecific variation in protein content.

3.1.2 | Protein estimation

Nitrogen content for CP calculation was measured using the Dumas combustion method with a Carlo Erba NA-1500 CNS analyser (Carlo Erba Instruments, Italy), at Georgia University Stable Isotopic Ecology Laboratory. In the first experiment, we tested 10 technical replicates per species. In the second experiment, all 48 isopods were measured separately. We used the popular 6.25 nitrogen-to-protein conversion factor.

Prior to the colorimetric quantifications, proteins were extracted by adding 0.5 M NaOH, sonication for 15 min at room temperature, and another sonication for 15 min at 80°C. Samples were then centrifuged for 10 min (15,000 RCF, 22°C), and the supernatant was removed. The solution was used for both colorimetric methods and diluted by a factor of 7 with DDW to be within the dynamic range of the calibration curves. Notably, there is no clear consensus in the literature regarding the preferred extraction method. Like all extraction methods, the method used may suffer from different caveats such as incomplete protein extraction and low stability until assay. Yet, we chose this method because our literature survey revealed that it is very popular in extracting proteins from a wide range of organisms such as fish, shrimps, grasshoppers, algae and grasses (Burkepile et al., 2006; Clissold et al., 2009; Cruz-Rivera & Hay, 2000). Since our goal was to compare the different quantification techniques and not the absolute protein content, the use of one extraction method over another (which deserves its own discussion) should not affect the results.

For both colorimetric methods, we used the commercial Pierce™ Protein Assay Kits (Thermo Scientific, No. 23200, and 23225 for Bradford and BCA, respectively). We followed the 'standard microplate protocol' for the Bradford assay and the 'microplate procedure' for the BCA assay. For both methods, we used two protein standards, BSA and bovine gamma globulin (BGG) (Thermo Scientific, No. 23209, and 23212, respectively). Thus, a single replicate within each method consisted of a 96-well plate, which included a series of eight dilutions of each protein standard (25–2,000 μg/ml), a blank, and the samples, all tested in triplicates. This setup allowed us to calculate the protein content of each sample based on the two protein standards and examine if, and how, they affect the results. Additionally, we tested if the type of calibration method (e.g. linear or quadratic curves), which is used for correlating between absorbance level and protein content, impacts the results. We used 10 replicates (plates) for each colorimetric method.

Following the Bradford protocol, we added 250 μl of the reagent to every 5 μl of the standard proteins and samples. We then manually lightly swirled the plate and directly started the absorbance measurement using a microplate reader (Epoch BioteK, USA). The plate was monitored at $\lambda = 595$ nm and $\lambda = 450$ nm for 22 min in 2-min intervals, that is, 12 consecutive absorbance measurements. We recorded the time since adding the reagent until the first measurement rather than incubating the plates for 10 min at RT, as suggested by the protocol. This allowed us to measure a single absorbance for each plate exactly 10 min after reagent addition. Furthermore, it enabled us to explore absorbance changes over time, and the consequences of these changes in determining protein content.

Following the BCA protocol, we added 200 μl of the reagent to 25 μl of the standard proteins and samples. The plate was incubated at 37°C for 30 min and then measured at $\lambda = 562$ nm. We used the same sampling timeline as for the Bradford assay. For statistical analyses, see Appendix S4.

3.2 | Results

Protein content differed greatly between quantification methods as well as between species ($F_{2,207} = 5{,}057.31$, $p < 0.001$, and

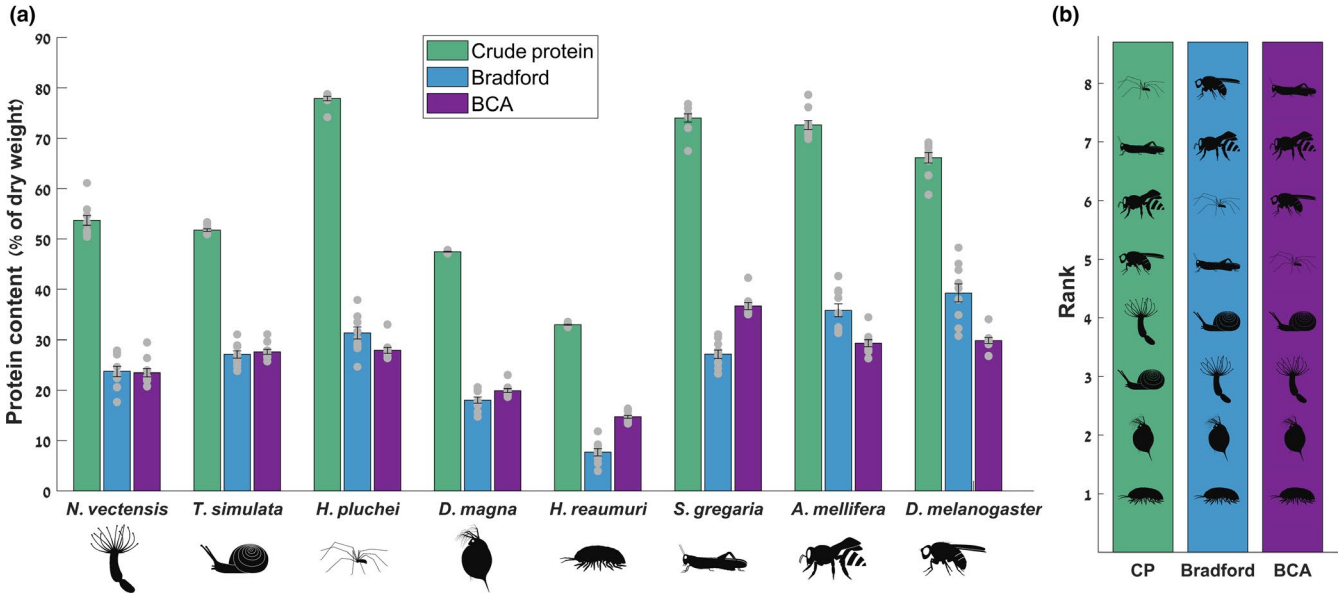

FIGURE 1 (a) Protein content (mean ± *SE*) of tested species, quantified by the three quantification methods. For the colorimetric methods, data calculated using BSA protein standards and linear calibration curves. (b) Ranking of the eight species by their total protein content estimated by the three quantification methods

$F_{7,207}$ = 535.41, p < 0.001 respectively; Figure 1a), with a significant interaction between these two factors ($F_{14,207}$ = 65.97, p < 0.001; Figure 1a). Crude protein estimations were about two-fold higher than those produced by colorimetric methods. Ranking the eight taxa by their total protein content revealed different taxonomic order for each quantification method (Figure 1b). The Pearson's correlation between CP and total protein estimations that were based on Bradford (r = 0.78) and BCA (r = 0.81) was higher than the correlation between the two colorimetric methods (r = 0.72).

The type of protein standard used and the interaction between the protein standard and the quantification method significantly affected the total protein estimations ($F_{1,279}$ = 671.14, p < 0.001; $F_{1,279}$ = 1,605.14, p < 0.001, respectively; Figure 2). Within the Bradford method, BSA yielded a lower protein estimation relative to BGG, while the opposite was true for the BCA method (Figure 2). The three-way interaction between the species, method and protein standard was also significant ($F_{7,279}$ = 8.792, p < 0.001), reflecting the different relationships between the method and the protein standard across the eight species. Notably, the two methods yielded very different results (about 2.5-fold difference) when using BGG, but more similar results when using BSA.

Protein estimations differed based on the calibration method used in both the Bradford ($F_{3,567}$ = 90.26, p < 0.001), and BCA ($F_{2,423}$ = 86.23, p < 0.001; Appendix S5, Table S4) methods. For both methods, the interactions between the standards and calibration methods were also significant ($F_{3,567}$ = 4.302, p = 0.005, and $F_{2,423}$ = 3.103, p = 0.046; for Bradford and BCA respectively). In the Bradford method, an interaction between the calibration method and the species was observed ($F_{21,567}$ = 1.955, p = 0.007, and $F_{14,423}$ = 1.415, p = 0.142; for Bradford and BCA respectively). In BCA, the use of higher-order polynomial curves gave rise to lower protein content results (see Appendix S5, Table S4), with relatively minor differences between the different curves. Similar to BCA, the linear curves tended to produce the highest protein content values also in the Bradford method. Yet, in the Bradford method, the use of different calibration methods gave rise to much larger variation in protein estimations than in BCA. (mean range of 10.78% for Bradford compared to 2.1% for BCA; Appendix S5, Table S4).

Incubation time significantly affected the protein content in five out of the eight Bradford techniques (i.e. combinations of quantification methods, standard proteins and calibration methods), and in all the six BCA techniques (Table 2). With few exceptions, the BCA-based protein estimations constantly rose over the 22 min of measurement. The mean rate of change was 0.38% per minute when using the linear curve, and 0.1% per minute when using the two higher-order curves. In the Bradford-based techniques, the protein estimations changed in both directions throughout the measurement, and typically faster than in the BCA. For instance, the protein content of the honey bee, measured by the common Bradford-BSA-linear technique, increased with time in half of the replicates (up to a rate of 0.33% per minute) and decreased in the other half (up to a rate of 0.47% per minute). Except for one technique (Bradford-BSA-quadratic), when the protein content significantly varied through time, it did so differently for different species (Table 2).

The different techniques' precision varied substantially (Table 2). Most of the variation is explained by the principal method, with much smaller and inconsistent effects of the protein standard and calibration method. The coefficient of variation (CV) of the Bradford-based techniques varied between 11.44 and 14.59 and was much higher than the BCA-based techniques that ranged from 4.2 to 7.7. The techniques that use BSA standard and a linear curve were moderately precise for both

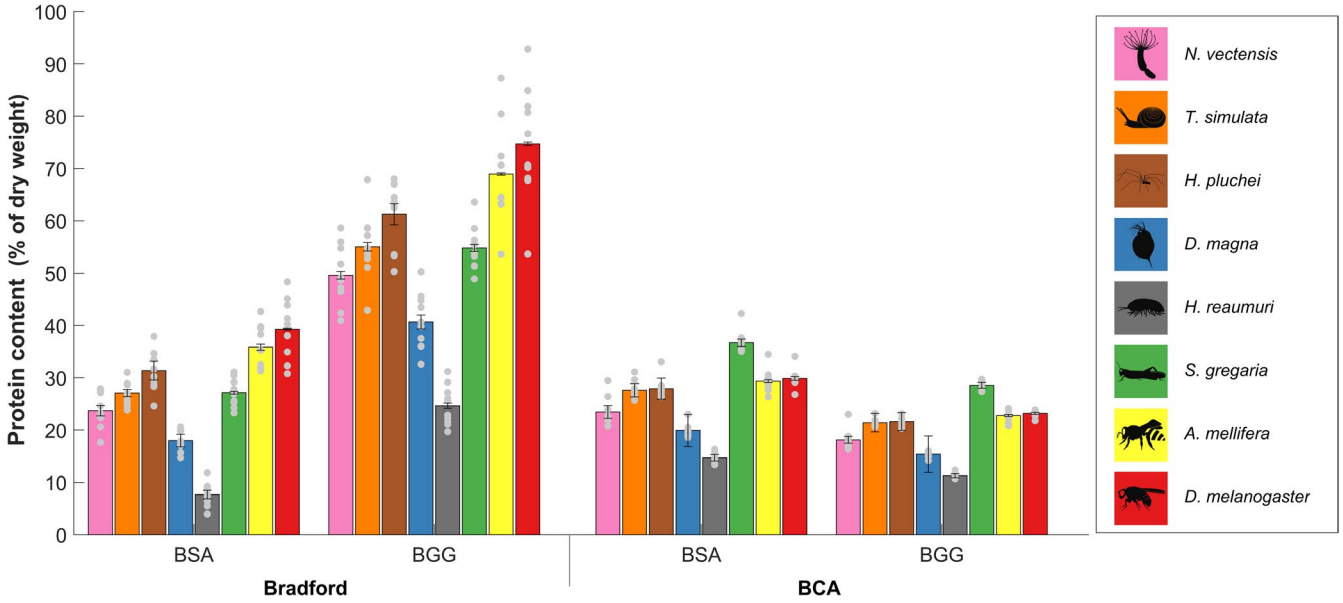

FIGURE 2 Protein content (mean ± *SE*) of the eight species, quantified by the Bradford and BCA methods, using both BSA and BGG protein standards for each method. Data calculated using linear calibration curves

Method	Standard protein	Calibration curve	A		B
			p-value: Time	*p*-value: Species-time	CV
Crude	–	–	–	–	2.83
Bradford	BSA	Linear	0.138	0.725	13.17
		Linear—ratio 595:450	<0.001	<0.001	12.36
		Quadratic	<0.001	0.277	14.38
		Cubic	0.573	0.716	13.79
	IGG	Linear	0.311	0.792	12.89
		Linear—ratio 595:450	<0.001	<0.001	14.59
		Quadratic	<0.001	<0.001	12.08
		Cubic	<0.001	<0.001	11.44
BCA	BSA	Linear	<0.001	<0.001	6.89
		Quadratic	<0.001	<0.001	5.24
		Cubic	<0.001	<0.001	7.70
	IGG	Linear	<0.001	<0.001	4.20
		Quadratic	<0.001	<0.001	5.64
		Cubic	<0.001	<0.001	5.32

TABLE 2 Empirical results for the different quantification techniques. (A) Statistical results of the ANCOVA models testing the effect of time on the protein content calculation in colorimetric methods. *p*-values of the co-variate 'Time' and its interaction with the fixed factor 'Species' are presented. (B) The mean of the coefficient of variation (CV) of the eight species

Bradford (CV = 13.17) and BCA (CV = 6.89). The highest precision was obtained by the CP method, with a CV of 2.83.

Different quantification techniques produced inconsistent results also when comparing conspecific isopods that were fed different diets (Figure 3). While the CP method and all six BCA-based techniques revealed a significant diet effect on the isopods' protein content, six out of the eight Bradford-based techniques revealed no differences (Appendix S5, Table S5). Furthermore, the post-hoc comparison results are not consistent, even across the techniques that yielded significant differences. No pair of diets caused significant differences in isopod protein content by all quantification techniques (Appendix S5, Table S5). Pearson's correlations between the isopods' body protein estimations using the CP method and the two common colorimetric techniques that used BSA standard and linear curve were relatively low (Bradford: $r = 0.47$; BCA: $r = 0.74$). The correlation coefficient between the two colorimetric techniques was 0.62.

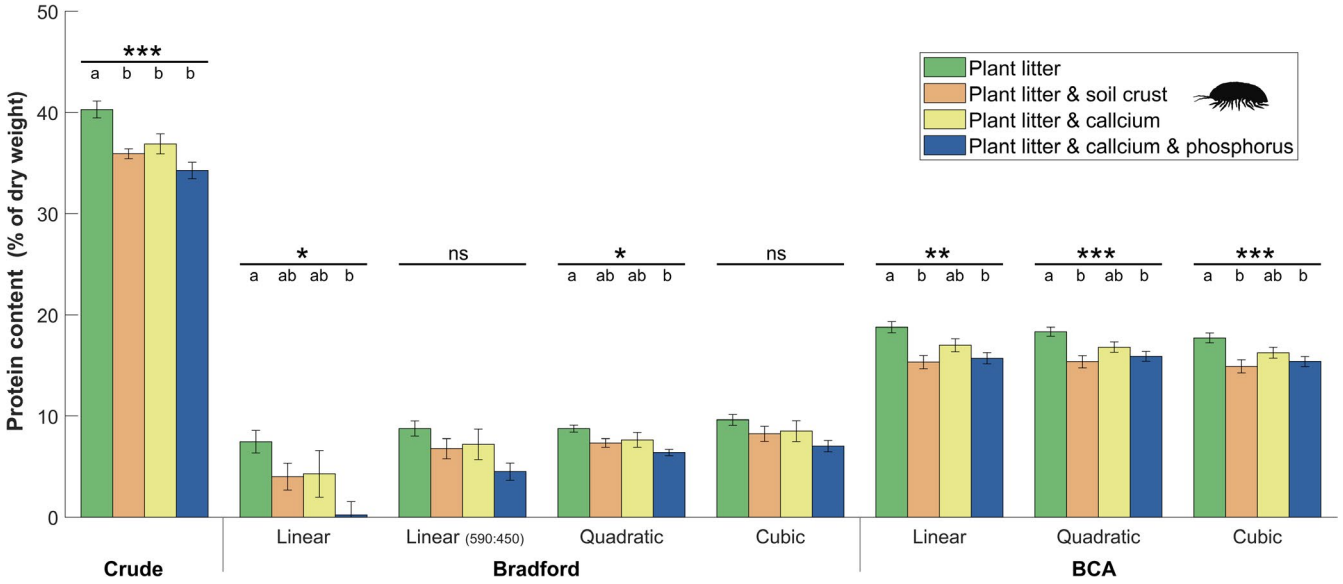

FIGURE 3 Protein content (mean ± *SE*) of desert isopods fed four different diets, as quantified by the CP, Bradford, and BCA methods. For the two colorimetric methods, data is calculated using the BSA protein standard. Significance levels for each technique are represented by asterisks ($^{ns}p > 0.05$; $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$), and different letters represent significant differences between treatments

4 | DISCUSSION

Ecologists use many protein quantification techniques that produce inconsistent results across taxa, and even between conspecifics. Our search of the ecological literature revealed 19 principal protein quantification methods, among which CP and Bradford assays were the most common. We also found large variations in the protocol details of the different principal methods. Most studies lacked sufficient information to allow meaningful assessments of the methodology, let alone to repeat the protocol in future studies. Our total protein quantification of eight phylogenetically diverse taxa using various CP, Bradford and BCA protocols revealed up to fourfold differences in the total protein estimation for a given sample. The differences in protein content estimations were inconsistent in magnitude across taxa, yielding qualitatively different taxonomic ranks for each method. We also demonstrated that Bradford, and to a lesser extent BCA, are sensitive to protocol changes. Inconsistencies in protein estimation when using the three principal quantification methods, and their derived techniques, were also evident when comparing the total protein content of desert isopods fed different diets, yielding qualitatively different answers to whether isopod protein content is affected by diet.

Our literature review revealed 19 different principal quantification methods used by ecologists to estimate total protein content, with the most prevalent being Bradford (34%). Other popular quantification methods were CP (26%), which was found to be the most precise method among the three examined (Table 2), and the Lowry assay (14%). The popularity of these methods in recent ecological studies was surprising, given the overwhelming appreciation in other disciplines that Bradford and Lowry assays are inferior to the BCA method. BCA has higher stability under alkali conditions, and better tolerance to interference compounds than the Lowery assay (Walker, 2009). Also, BCA is more precise than the Bradford assay as our empirical measurements showed. Likewise, our search revealed that ecologists rarely used the AAA. This method was used only in 0.48% of the studies examined, despite a growing recognition in other scientific fields that it may produce more accurate total protein estimations than alternative methods (Angell et al., 2016; Mishyna et al., 2019).

The numerous principle quantification methods used by ecologists poses major concerns. Protein quantification by different methods yields different estimations depending on the specific protein composition, and the existence of interfering agents and other nitrogenous compounds (Olson & Markwell, 2007). Indeed, we found that different methods lead to large differences in total protein estimations reaching up to 6.7-fold differences for a single replica, and up to 4.3-fold differences for the average protein estimation per taxon. The correlation coefficients between methods ranged from 0.72 to 0.81 when examining mean protein estimation across taxa, and became weaker (0.47–0.74) for samples of the same taxa. Our findings are consistent with studies outside the field of ecology comparing the performances of these and additional methods (e.g. Lowry, UV absorbance, AAA). These studies found similar or even higher variations in protein estimations, as well as much lower, or negative correlations between methods (Okutucu et al., 2007; Seevaratnam et al., 2009). For instance, when comparing protein quantification of several food sources by different methods, Nwachukwu and Aluko (2019) found negative correlations between different methods, such as the Bradford assay versus AAA or the Lowry assay versus the O-phthaldialdehyde fluorometric protein assay. Consequently, comparisons of absolute protein content using different principal methods are unreliable.

Even more troubling was our finding that variations in protein estimations are inconsistent across taxa. We found that the ratio

between total protein estimations by the three quantification methods differed across species. For example, the BCA method yielded higher protein content for grasshoppers and isopods than did the Bradford method and conversely, the protein estimations by Bradford was higher for honey bees and for fruit flies (Figure 1). Similarly, the isopod protein content was 69% lower when quantified by Bradford than by CP, but the fruit flies protein content was only 21% lower when quantified by Bradford than CP. These taxa-specific differences suggest that it is impractical to find a conversion factor that corrects for protein estimation differences by different quantification methods across taxa. Therefore, ranking species by their protein content is a questionable practice when using a single quantification method, and even more so when using estimation produced by different methods, making protein measurements in the food-web context problematic.

We found that 91% of the studies assessing an organism's protein content did so to reveal how a single taxa's protein content was affected by environmental conditions, focusing on relative, rather than absolute protein estimations. This prevalent experimental approach may be considered less sensitive to methodological biases than inter-taxa comparisons. However, we found that different protein quantification protocols yielded qualitatively different results when comparing the body composition of isopods fed different diets. Several Bradford protocols revealed no differences in body composition, while BCA protocols and CP estimations showed significant differences. We found qualitative differences in the results of the post-hoc comparisons between groups, even when using different BCA protocols. Our findings suggest that scientific inferences based on such intra-taxa comparative studies should be evaluated with caution.

Our literature search revealed that ecologists use a multitude of different protocols for each principle method. We empirically demonstrated that such nuanced procedural changes can produce distinct protein estimations. Yet, it is apparent that many ecologists are unaware of this potential problem, given the inadequate protocol reporting by most studies (Table 1).

Within the colorimetric methods, the standard protein had the largest effect on protein estimations, leading to up to 3.2-fold differences. Nevertheless, the standard was reported in only 57% of the studies. The calibration method had a smaller yet significant effect on protein estimation, and this was also affected by the standard used (both methods) and taxa (only Bradford). Assuming a linear relationship between protein content and absorbance is problematic for both methods (Olson & Markwell, 2007; Walker, 2009; Zor & Selinger, 1996), but remains the most common practice in ecological research. In both methods, the incubation time also affected protein estimation, which increased or decreased with time, varying by species. Interestingly, the key role incubation time plays in determining protein estimations was discussed in the two original papers (Bradford, 1976; Smith et al., 1985). Yet, as our literature review clearly showed, this important source of variation seems to be frequently ignored by ecologists.

The way ecologists estimate CP is also inconsistent. Our literature review revealed that the Dumas combustion and the Kjeldahl methods are used in roughly similar proportions (44% and 56%, respectively). Findings from other fields show that Dumas combustion produces 1.5% higher N estimations than Kjeldahl methods (Daun & DeClercq, 1994; Thompson et al., 2002). This difference can lead to a 7.2%–9.4% difference in protein estimations, using the common conversion factors. Despite this dependency, only 78% of the CP using papers reported what N–determination method they used.

Our CP estimations were consistently higher across all taxa relative to the two colorimetric method estimations. This is likely because we used the 6.25 conversion factor. This popular factor, which dates back to the 19th century, assumes that proteins are 16% nitrogen and that proteins are the only nitrogenous compounds in the sample (Mariotti et al., 2008). These assumptions are false for most organisms. Thus, it is surprising that despite our dataset's diversity of organisms and the well-founded criticism in other scientific fields for the indiscriminate use of the 6.25 factor (Mariotti et al., 2008; Moore et al., 2010; Sosulski & Imafidon, 1990), 89% of the ecological CP studies that reported the conversion factor, used it. Other studies used conversion factors ranging from 4.78 to 6.08. Yet, we argue that regardless of the calibration method used, no single conversion factor is possible across species due to the very large inter-specific variation in protein composition and non-protein nitrogenous content.

Researchers in other disciplines, such as agriculture and food sciences have proposed taxa-specific conversion factors to improve the CP accuracy. These taxa-specific factors are based either on calibration by colorimetric methods (e.g. González López et al., 2010; Slocombe et al., 2013) or AAA (e.g. Lourenço et al., 2002; Mishyna et al., 2019). As previously discussed, colorimetric methods are sensitive to variation in protein composition (Olson & Markwell, 2007) and interference from other non-protein compounds (Compton & Jones, 1985; Walker, 2009), and produced inconsistent results across species. Consequently, colorimetric methods cannot be considered a reliable way to produce a species-specific conversion factor. AAA is considered a more accurate quantification method than the indirect alternatives, hence is increasingly used as a species-specific CP calibration method (Mæhre et al., 2018). As abovementioned, AAA also suffers from several biases mostly due to amino acid degradation or imperfect breakdown of peptide bonds in the hydrolysis phase (Krul, 2019; Mariotti et al., 2008; Mosse, 1990; Rutherfurd & Gilani, 2009). Yet even if we assume high accuracy, intra-specific variation in non-protein nitrogenous compounds may hinder attempts to develop species-specific conversion factors. For instance, Mæhre et al. (2018) used AAA to estimate the total protein of five food types. They then measured the N content and multiplied it by published food-specific factors that were also calculated by AAA. The species-specific conversion factors led to 30% to 54% overestimation of the protein content relative to the AAA direct estimations. Consequently, it seems that regardless of the calibration method,

species-specific nitrogen-to-protein factors cannot be considered a reliable method for protein estimations across species and even between conspecifics.

The consequences of our limited ability to link protein content and N content extends beyond issues with calculating CP. Ecological stoichiometry, for example, assumes high correlation between N and total protein content (Sterner & Elser, 2002). However, a growing number of recent studies find only weak association between protein and N contents (Mæhre et al., 2018; Nwachukwu & Aluko, 2019; Rinehart & Hawlena, 2020; Wilder & Jeyasingh, 2016). Our results concur with this general trend. We found a moderate association between N estimations and total protein estimations that were based on Bradford ($r = 0.78$) and BCA ($r = 0.81$) across species, but even weaker correlation when comparing individuals of the same species (Bradford: $r = 0.47$; BCA: $r = 0.74$). We suggest that this apparent deviation from ecological stoichiometry's core principals may stem from other nitrogenous compounds, such as chitin (which contribute to the total N content but were not accounted for) as well as the methodological difficulties involved in accurately and precisely measuring total protein (e.g. Van Dievel et al., 2016). Regardless of the exact reason, these difficulties may hinder attempts to integrate concepts of organismal and ecosystem ecology. For instance, an emerging area of ecological research focuses on predicting how food-web interactions effect ecosystem processes, such as nutrient cycling (Hawlena & Schmitz, 2010). Such integration requires converting changes in organismal nutrient balance, measured as biomolecules, to elements using ecological stoichiometry principles. Thus, failure to accurately convert total protein estimations to N may impede our ability to integrate these fields—limiting our understanding of critical links between biodiversity and ecosystem function.

4.1 | Guidelines for protein quantification

To encourage better and more comparable total protein determination in ecological research, we provide guidelines on preferred quantification protocols and how to report the methodology and results. Currently, a non-biased method for quantifying total proteins does not exist. AAA is a more direct method for quantifying proteins and is often considered superior to indirect methods by practitioners in neighboring fields (Mariotti et al., 2008). Unfortunately, as of today, AAA is immensely expensive and time consuming, requires specific equipment, and cannot be routinely used in ecological research were numerous samples are being quantified in search for inter- and intra-specific variation. Moreover, attempts to use AAA as a species-specific calibration tool for other quantification methods seems unreliable. This is probably why <0.5% of the ecological studies that quantified protein in the last 10 years used AAA. Thus, until AAA becomes less expensive and more accessible to ecologists, we recommend using a specific BCA protocol (see Appendix S6). This will guarantee consistent results across ecological studies and improve the precision and accuracy of total protein estimations. BCA is fast, affordable, and requires basic equipment and elementary laboratory expertise. It is more precise, has higher stability under alkali conditions and better tolerance to the presence of interference compounds than alternative methods, and is only moderately affected by the sample's amino acid composition and incubation time than comparable methods (Olson & Markwell, 2007; Smith et al., 1985; Walker, 2009).

5 | CONCLUSIONS

Protein quantification is a routine procedure in ecological studies despite the well-accepted limitations of protein determination methods that are largely overlooked by ecologists. Our literature search revealed that ecologists use a large number of protein quantification procedures, often without reporting crucial information to allow evaluating or repeating those procedures. Moreover, our empirical work demonstrated that various quantification methods, and their derived protocols, yield different protein estimations that are inconsistent in rank across taxa, thus preventing conversions between protocols and the use of these data for between-study comparisons (e.g. metanalyses). Most ecological studies quantify proteins to compare conspecific responses to various environmental conditions. We found that different quantification methods give qualitatively different results, questioning the robustness of this common experimental approach. To avoid these pitfalls, we recommend adopting a common BCA-based protocol that seems superior to alternative methods, until more accurate and affordable methods are accessible. Notably, we focused our experimentation on animal samples. Yet, we have no reason to suspect that plant or any other biological samples that vary in composition of amino acids, non-protein nitrogenous compounds, and interfering substances will not suffer from similar methodological drawbacks. We want to emphasize that our goal is not to undermine the use of protein measurements in ecology. On the contrary, we believe that accurate and precise protein quantification is imperative for many ecological subfields. We hope that our study will shed light on this methodological issue and hopefully limit the impact of protein quantification's caveats on ecological theory.

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AUTHORS' CONTRIBUTIONS

M.Z. and D.H. conceived the project. All authors designed the study and performed the literature survey; M.Z. and S.K. performed the empirical protein analyses; M.Z. analysed the data. All authors wrote the manuscript.

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SUPPORTING INFORMATION

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