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Original Articles

Cellular energy allocation analysis of multiple marine bivalves using near infrared spectroscopy

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ABSTRACT

This paper proposes an alternative procedure for calculating Cellular Energy Allocation (CEA) in marine bivalve species. CEA is a measure of the difference in total energy available and energy consumption and is used to assess the health of organism fitness for growth and reproduction. In this study near infra-red spectral (NIRS) quantitative data-driven modelling techniques were applied to total energy available (total protein, lipid and glycogen content) and energy consumption (electron transport system activity (ETS)) of five marine bivalve species (*Saccostrea glomerata*, *Ostrea angasi*, *Crassostrea gigas*, *Mytilus galloprovincialis* and *Anadara trapezia*) to produce models for measuring each bioenergetic component and calculating CEA, a biomarker of energetic stress response. Comparison of predicted to measured data showed low prediction errors (RMSEP) and high ratio of predictive error to inter-quartile distance (RPIQ) values. RMSEP for protein, lipid, glycogen and ETS models were 8.7%, 6.8%, 7.6% and 12% of the dynamic range of the validation datasets respectively while RPIQ were 3.6, 5.2, 4 and 3.7 respectively. Conversion of model outputs to energetic equivalents and calculation of total energy stores as per the CEA method demonstrate a close relationship between measured and modelled results (105% ± 3%). These metrics indicate that the four energy models are accurate, robust and reliable for quantitative application to all five species within the sample variation of the energetic storage measures. Applying NIRS quantitative models using freeze dried tissue eliminates water interference in NIRS capture and provides improved sample homogeneity for both spectral capture and chemical analysis. This technique enables simultaneous analyses of protein, lipid, glycogen and ETS activity to be achieved with a much higher throughput of samples (100–200 samples daily) than conventional energetic storage analysis techniques. In addition, the technique is non-destructive to tissue samples and freeze dried samples can be stored for additional analyses. The value of CEA as a response measure in *S. glomerata* are included, with examples of changes in CEA over four seasons and the response of oysters to a known metal contamination gradient.

1. Introduction

The assessment of energy allocation and use is emerging as an effective method for assessing organism response to multiple environmental stressors (Mouneyrac et al., 2012). It provides an early warning system to environmental stress as responses are usually rapid and/or sensitive to low contaminant concentrations (Moolman et al., 2007; Moore et al., 2006).

Cellular Energy Allocation (CEA) is a physiological energetic biomarker and can provide quantified data on an organism's energetic response to stress (Smolders et al., 2004). It assesses changes in the total energy stores available (E_a) (total glycogen, protein and lipid content) and the energy consumption (E_c), estimated by measuring the enzymatic reaction for electron transport system activity (ETS). ETS is linked to the cellular respiration rate or the oxygen consumption process. The difference between E_a and E_c represents the net energy budget and is reported as CEA (De Coen and Janssen, 1997; De Coen and Janssen, 2003; Moolman et al., 2007; Verslycke et al., 2004).

Bivalve species inhabiting estuarine and coastal environments have been used extensively for environmental monitoring as they are filter feeders, sessile, relatively long-lived and tolerant to environmental stressors (Anacleto et al., 2015). Bivalves also have significant economic value, with oyster, mussel and other mollusc aquaculture industries globally worth US$19 billion (FAO, 2016). Bivalve species have exhibited changes in energetic composition in response to changes is physiochemical conditions and anthropogenic changes (Anacleto et al., 2015; Aranda et al., 2014; Baek et al., 2014; Bi et al., 2016; Kumar et al., 2015; Li et al., 2009; Mendo et al., 2016; Múgica et al.,

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2015; Nahrgang et al., 2013).

Near Infrared spectroscopy (NIRS) modelling is now being employed as an alternative to traditional chemistry procedures for qualitative and quantitative analysis in food and agriculture quality evaluation due to its rapid, non-invasive data collection and analysis capabilities (Bellon-Maurel and McBratney, 2011; Wu et al., 2009). NIRS measures the absorbance of light in the near infrared (NIR) band (4000–10,000 cm^{-1}) using Fourier Transform Near Infrared Spectroscopy (FT-NIR). In the near-infrared range, absorption corresponds to overtones and combinations of fundamental bands of molecular vibrations (Bellon-Maurel and McBratney, 2011). Traditional chemistry methods for testing energetic components are time consuming and do not easily lend themselves to comprehensive studies with high numbers of samples (Brown, 2011). Data analysis of NIRS using multi-linear regression allows for computation of predictive models (Mata Sánchez et al., 2015; Xiaobo et al., 2010). In undertaking regression analysis, more effective and robust correlations are obtained by applying an approach to discriminate within the spectra on which band widths to use in the quantitative modelling (Xiaobo et al., 2010). At least 100 samples and preferably more are needed to develop NIR calibrations (ISO 12099, 2010(E)); in addition, an independent sample set is needed to test calibrations (Reeves and Smith, 2009), with at least 20 samples being used (ISO 12099, 2010(E)).

NIRS has been used in previous studies to quantify protein changes (Barth, 2007), carbon isotope characteristics (Melton et al., 2013; Miller et al., 2004), and biochemical composition in a range of biological materials including bivalves (Brown, 2011; Cunha Junior et al., 2015; Eskildsen et al., 2014; Kucheryavskiy and Lomborg, 2015; Maniwara et al., 2014; Mata Sánchez et al., 2015; Pan et al., 2015). Soil enzyme activity has also be undertaken using NIRS quantitative modelling (Dick and Aditi, 2011).

Quantitative modelling using NIRS has been undertaken previously to determine protein, lipid and glycogen concentrations of wet tissues for two oyster species (*S. glomerata* and *C. gigas*) (Brown, 2011) but these have suffered from several severe limitations. These are: difficulty in homogenising wet bivalve tissue; water presence causing interference in spectral capture (Aenugu et al., 2011) and baseline shifts for the rest of the spectra due to light scattering (Kucheryavskiy and Lomborg, 2014) and knowledge based bandwidth selection methods which are not optimal and introduce operator bias (Xiaobo et al., 2010). These limitations have been addressed in this study and a companion study (Bartlett et al., 2018) by freeze drying the tissue to eliminate water interference and improve homogenisation and using data-driven bandwidth selection methods. In addition, alternative biochemical methods were employed to improve reliability. Enzyme activity has been shown to be retained in freeze dried tissue (Sundari and Adholeya, 2000).

This paper presents procedures for using NIRS quantitative modelling with wholly data-driven methods incorporating GA bandwidth selection and GA-PLS to determine the protein, lipid and glycogen content and ETS activity of five bivalve species (*Saccostrea glomerata, Ostrea angasi, Crassostrea gigas, Mytilus galloprovincialis* and *Anadara trapezia*) combined in a single model to undertake CEA analyses. Five species have been included to extend the range and potential application of models.

2. Materials and procedures

2.1. Sample collection

Bivalve samples were collected over a period of 15 months from four New South Wales field sites, two Victorian field sites and one site at Port Lincoln, South Australia (Bartlett et al., 2018).

Bivalves were opened and whole wet weights determined then frozen at −80 °C prior to being lyophilised using a LABCONCO freeze dryer. Samples were freeze dried for 48 h and re-weighed to determine moisture loss, then ground into a fine powder (< 20 μm) using an analytical mill (230v A11 Basics S5 IKA mill). Sample NIR spectra were captured within 24 h then samples were stored in a desiccator until chemical analysis.

2.2. Near Infra-red spectra (NIRS) collection

NIRS were collected with a Perkin Elmer Frontier FT-IR Spectrometer using the NIR spectral unit. NIR spectra were captured at wavenumbers from 10,000 to 4000 cm^{-1} measured as absorbance units at a resolution of 16 cm^{-1} with data intervals of 2 cm^{-1} with 32 scans. NIRS capture was undertaken in triplicate using a 30 mm dish with samples rotated up to 120° between each image capture. Spectra were captured using Perkin Elmer Spectrum software, v.10.4.3.339. Samples added to the dish were gently pressed into the dish then tapped three times with a spatula to ensure even packing and distribution. Spectra were corrected for stray light and reference corrected.

2.3. Chemical and enzymatic analysis

2.3.1. Total glycogen and protein concentrations

Total glycogen and protein concentrations were measured using 0.02 g of freeze dried tissue re-hydrated in 5 mL of deionised water. Trichloroacetic acid 15% (w/v) (TCA) was added to a ratio of 3:1 (v/v/v) tissue to TCA then incubated for 20 min at −20 °C (Rajalingam et al., 2009). Extracts were then centrifuged at 5000 × g for 10 min at 4 °C. The supernatant was removed for glycogen analysis and the pellet retained for protein determination. Glycogen was determined by incubating 100μL of supernatant with 200 μl of 5% w/v phenol and 800 μl of 18 M H_2SO_4 at 25 °C for 30 min (DuBois et al., 1956); 150 μL was measured colorimetrically at 492 nm in triplicate and using glucose as a standard. Protein was determined by digesting the pellet in 500 μL 1 M NaOH for 30 min at 60 °C. The sample was allowed to cool, then 300 μL of 1.67 M of HCl was added to neutralise the samples. Protein was determined using Bradfords reagent with bovine albumen serum as a standard and measured colorimetrically at 590 nm (Bradford, 1976).

2.3.2. Total lipid concentration

Total lipid concentration was measured using the 2:1 w/v chloroform/methanol extraction method (Folch et al., 1956). In brief, chloroform, methanol and 0.88% (m/v) NaCl was added to 0.3 g of dried tissue samples to obtain a tissue to solution ratio of 1:20 (m/v) and a ratio of 8:4:3 (v/v/v) of chloroform/methanol/water. Samples were agitated for 10 min (Intelli Mixer RM2M, 10 min, 35 rpm) then centrifuged at 5000 × g for 5 min. Supernatants were removed and pellets re-extracted to ensure complete lipid recovery (Dickinson et al., 2012). The supernatants were combined then centrifuged at 1000 × g for 10 min and a sub-sample of the lower chloroform phase removed. The chloroform sub-sample was mixed with H_2SO_4 and charred at 200 °C for 15 min, then diluted with 2 mL of water and measured colorimetrically at 370 nm using tripalmitin as a standard (De Coen and Janssen, 1997).

2.3.3. Electron transport system activity (ETS)

A slightly modified version of previously published ETS methods was used to determine maximum energy usage potential (De Coen and Janssen, 1997; Owens and King, 1975). Briefly, freeze dried tissue was homogenised in 750 μl of cold homogenising buffer (0.1 M Na_3PO_4 buffer, 75 μM MgSO4, 15% (w/v) polyvinyl pyrrolidone and 0.2% (v/v) Triton-x 100) for 2 min then centrifuged at 4 °C for 10 min at 8500 × g (Owens and King, 1975). One part supernatant was then added to a micro plate and mixed with 3 parts cold substrate solution (0.1 M Sodium phosphate buffer, 1.7 mM NADH, 0.25 nM NADPH and 0.2% (v/v) Triton-x 100) (Owens and King, 1975) and 1 part room temperature reagent solution (8mM 2-p-iodo-phenyl 3-p-nitrophenyl 5-phenyl tetrazolium chlorine (INT)) (De Coen and Janssen, 1997). The microplate