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## GLYCOGEN CONCENTRATION IN FREEZE-DRIED TISSUES OF EASTERN OYSTER (*CRASSOSTREA VIRGINICA*) USING NEAR INFRARED REFLECTANCE SPECTROSCOPY TO DETERMINE THE RELATIONSHIP BETWEEN CONCENTRATIONS OF THE TISSUES EXCISED FOR HISTOLOGICAL SAMPLING AND THE REMAINING TISSUES

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**ABSTRACT** To improve the accuracy and reproducibility of the previous near infrared reflectance spectroscopy (NIRS) model for glycogen in the oyster species *Crassostrea virginica*, a new model using freeze-dried samples was developed. The NIRS glycogen calibration model was developed using 380 individual oyster samples collected between 2014 and 2016 from several locations in the Chesapeake Bay. Homogenized freeze-dried samples were scanned in the near infrared region between 1,000 and 2,500 nm. In parallel, glycogen concentration (GC), measured as percent dry weight, was determined using laboratory-based methods. The two sets of data allowed us to build a NIRS model based on freeze-dried oyster meats, and the model gave a strong prediction of GC [coefficient of determination for validation ( $R^2_{\text{val}}$ ) = 0.96 and residual predictive deviation (RPD) = 5.2]. The second part of the study applied the model to determine GC among 39 diploid and 40 triploid *C. virginica* and determined the strength of the relationship between the GC of tissues excised for histological sampling to the remaining tissue (*corpus*) to verify assumptions made throughout the literature. There was an estimated  $R^2 = 0.99$  between the GC in the *corpus* and the tissues of whole oyster meat. Among the samples, two factors, ploidy and size (shell height), had a significant effect on GC.

**KEY WORDS:** chemical analysis, near infrared reflectance spectroscopy, oyster, *Crassostrea virginica*, glycogen concentration, freeze-dry

### INTRODUCTION

The eastern oyster *Crassostrea virginica* (Gmelin, 1791) is currently an important aquaculture species on the East Coast of the United States of America where 95% of the production occurs (FAO 2014). The success of oyster culture is mostly because of the development of large-scale intensive aquaculture using mass selection and polyploidy improvement (Frank-Lawale et al. 2014). Currently, in Virginia, triploid oysters represent 93% of all farmed oysters (Hudson & Murray 2014). In oyster species, genetic improvement through triploidy leads to a higher growth rate (Garnier-Gere et al. 2002, Nell & Perkins 2005) and reduced fecundity (Mann 1979, Allen & Downing 1986, Jouaux et al. 2010). These two characteristics give economic advantages and superior market quality over diploid oysters (Nell & Maguire 1998, Nell 2002, Guo et al. 2009). Superior meat quality is often linked to higher concentration of glycogen in triploids compared to diploids. In fact, glycogen has been linked to texture (Maguire et al. 1995) and flavor enhancement, more particularly the umami taste (Konosu et al. 1974, Sakaguchi & Murata 1989, Mason & Nell 1995, Hong et al. 2002). Moreover, in oyster species, glycogen has also been linked to the survival and success of reproduction (Gabbott & Stephenson 1974, Deslous-Paoli & Heral 1988). For all these reasons, an accurate, reproducible, and efficient method to measure glycogen concentration (GC) in oysters may provide a means of routine quantification of this key trait in oyster species.

Traditionally, quantification of biochemical parameters requires considerable time, money, and the use of chemicals that might be toxic to humans and the environment. Near infrared reflectance spectroscopy (NIRS) technology is a promising tool

that may provide a faster, safer, and more eco-friendly alternative to traditional analytical methods in quantifying biochemicals of interest. The principle of NIRS is based on absorption of energy from the IR spectrum by the target samples. The reflected light provides a spectral “fingerprint” of the composition of a sample and can be used to create quantitative models. Last year, the Aquaculture Genetics and Breeding Technology Center (ABC) developed two models suited for compositional analysis of moisture and glycogen in *Crassostrea virginica* (Guévelou & Allen 2016). The models were developed using homogenized tissues and showed a strong correlation between the predicted and measured data with a coefficient of determination for validation ( $R^2_{\text{val}}$ ) of 0.97 for moisture and 0.94 for glycogen. Recently, however, NIRS models developed on freeze-dried *Crassostrea gigas* tissues have claimed to reach even higher correlations and residual predictive deviation (RPD) values for glycogen (Wang et al. 2015). A NIRS model has not yet been developed using freeze-dried tissues of *C. virginica*. Therefore, one aspect of this study was to improve the accuracy of GC measurements in the eastern oyster *C. virginica* via NIRS by developing a model using freeze-dried oyster tissues.

The second part of this study applies the newly developed NIRS model to evaluate GC variation within and among *Crassostrea virginica* individuals. First, GC in two different anatomical sections of an oyster, a standard histological cross-section and the oyster meat remaining after dissection, were determined. In many oyster studies, sampling involves the dissection and histological analysis of a cross section of the visceral mass cut near the junction of the gills and the palps. This section, herein referred to as the *slab*, can be used for several quantitative and qualitative measurements. For example, the percent surface area occupied by the gonad provides an estimate of reproductive effort (Heffernan & Walker 1989,

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Royer et al. 2008), whereas some staining techniques show the level of specific gene expression or reveal the presence of pathogens (Montagnani et al. 2001, Stokes & Burreson 2001, Fabioux et al. 2004). Most of the time, the remaining part of the oyster, hereafter referred to as the *corpus*, is not kept for any further analysis, yet in some experiments the *corpus* has been treated as representative of the whole oyster (Arcos et al. 2009, Guévelou et al. 2013, Jeung et al. 2016). The extent to which the biochemical composition and, therefore, the glycogen of the oyster differs after the removal of the *slab* is unknown. The *slab* and *corpus* are anatomically different, as many organs are not included in the *slab* (e.g., adductor muscle and labial palps). In some cases, the *slab* may represent a significant fraction of the total oyster weight.

Using the *corpus* as a proxy for the entire oyster can enable associations between histological observations and biochemical measurements that may be important in physiological studies. Glycogen concentration is often used as an indicator of condition, or when examined over time, as evidence for effects of physiological processes, such as gametogenesis (Gabbott 1975, Mann 1979, Berthelin et al. 2000). Even with a strong correlation in the GC of the *corpus* and the entire oyster, other factors not specific to the condition of the oyster may have a significant influence on the *corpus* GC and confound results when not taken into account. One of these factors is the size of the oyster. To maintain sampling consistency, *slabs* for histology are often cut at a set width. The size of the *slab*, however, and more importantly the ratio of the mass of the *slab* to the total mass of the oyster, will increase with decreasing oyster size.

The main aim of the current study was to improve and significantly refine the NIRS glycogen model by freeze-drying and reanalyzing samples from Guévelou and Allen (2016) as well as measuring using additional freeze-dried oyster samples. To the best of our knowledge, this was the first attempt to develop NIRS calibration to predict the composition of *Crassostrea virginica* using freeze-dried samples. The second aim was to apply this new model to determine how accurate the *corpus* is in representing the GC of the whole oyster meat. Lastly, it was determined if ploidy, oyster size, and size ratio of the *corpus* to the *slab* had an effect on the GC of the *corpus*.

## MATERIALS AND METHODS

### Sample Collection for NIRS Glycogen Model

Eastern oysters (*Crassostrea virginica*) ranging in shell height (measured as maximum dimension between the hinge and the bill) from 50.3–123.8 mm and wet meat weight from 1.3–25.4 g were collected between January 2014 and December 2015 for the NIRS glycogen freeze-dry model. The collected oysters were derived from different lines, families, and ploidies (diploid, triploid, and tetraploid) cultivated by ABC within the Chesapeake Bay, USA (Table 1). For the NIRS glycogen model, the majority of the samples (206 oysters) were used in the previous study Guévelou and Allen (2016); from January 2014 to July 2015, wild *C. virginica* as well as *C. virginica* from several different domesticated lines developed by ABC were sampled

TABLE 1.

Top: Samples of *Crassostrea virginica* used in the calibration of the NIRS model on freeze-dried tissue. Bottom: Samples used for *slab/corpus/total glycogen concentration estimation*.

Line	Ploidy	Harvest date	Location	<i>n</i>	Wet meat weight (g)
DEBY11	2N	January-14	York River	25	13.0 ± 2.2
hANA11	2N	April-14	York River	24	17.9 ± 3.6
hANA11	2N	July-14	York River	23	10.8 ± 1.5
XB13	2N	October-14	York River	22	3.7 ± 1.5
DEBY13	2N	June-15	York River	25	6.5 ± 1.5
WILD	2N	June-15	York River	27	6.1 ± 2.0
GEN13	4N	July-15	York River	10	3.9 ± 1.0
GNL13	4N	July-15	York River	10	9.2 ± 2.8
DEBY13	3N	July-15	Cherrystone Inlet	10	11.3 ± 3.4
hANA13	3N	July-15	Rappahannock River	10	11.2 ± 2.7
DEBY13	2N	July-15	Milford Haven	10	8.1 ± 2.5
DEBY13	2N	July-15	York River	10	11.2 ± 2.3
DEBY13	2N	November-15	Milford Haven	20	10.9 ± 3.0
DEBY14	2N	November-15	Milford Haven	18	8.9 ± 1.8
Lola14	2N	November-15	Milford Haven	20	6.7 ± 2.3
DEBY13	2N	December-15	Milford Haven	20	10.4 ± 3.5
DEBY14	2N	December-15	Milford Haven	20	8.2 ± 1.8
Lola14	2N	December-15	Milford Haven	19	4.7 ± 1.1
DEBY13	2N	December-15	Milford Haven-hatchery	18	7.4 ± 1.7
DEBY14	2N	December-15	Milford Haven-hatchery	20	5.9 ± 1.2
Lola14	2N	December-15	Milford Haven-hatchery	20	4.6 ± 1.1
DEBY15	2N	August-16	York River	19	5.8 ± 1.8
XB13	2N	August-16	Rappahannock River	20	11.0 ± 2.3
DEBY14	3N	August-16	York River	20	14.4 ± 2.3
DEBY15	3N	August-16	York River	20	9.8 ± 3.2

Indicated are lines, ploidy, harvest date, location, number of samples (*n*), mean wet meat weight (g) ( $\bar{x}$ ), and standard deviation (SD) (numbers after line name indicate year the generation was created. WILD indicate oysters form the wild, the year of spawning is unknown.

from four different waterways around the Chesapeake Bay: York River, Cherrystone Inlet, Rappahannock River, and Milford Haven. In these samples, ploidies were assessed using flow cytometry as described by Matt and Allen (2014): triploid oysters DEBY13 and hANA13 were 100% triploids and tetraploids oysters GEN14 and GNL14 were 100% tetraploids, (Guévelou & Allen 2016). An additional 175 oysters were sampled exclusively for this freeze-dry model. In November and December 2015, three groups of diploid oysters were used from the DEBY and LOLA lines: DEBYs spawned in 2013, DEBYs spawned in 2014, and LOLAs spawned in 2014. In November 2015, 18–20 oysters of each group were sampled from Milford Haven (Mathews County, VA). The remaining oysters were separated and either kept in Milford Haven or held under controlled conditions at 4°C. After 1 mo, 18–20 oysters from each group at both locations were sampled (Table 1). Oyster meats sampled from January to October 2014 were shucked, weighed, transferred to resealable storage bags, and frozen at –80°C for 8–18 mo before analyses. Oysters sampled from June 2015 to December 2015 were shucked, weighed, transferred to a 60 ml polypropylene jar, and frozen at –80°C before analyses. All oyster meats were diced coarsely with scissors and then homogenized with an OMNI General Laboratory Homogenizer (OMNI International, Kennesaw, GA) for 60–120 sec.

#### Sample Collection for Glycogen Estimation in Slab and Corpus

A total of 39 diploid and 40 triploid oysters were collected in August 2016 to determine the GC in the *slab* and *corpus*, herein referred to as the *slab* experiment. The diploids were sampled from two lines: DEBY and XB from the York River and Rappahannock River, respectively. The triploids were sampled from the York River from two groups of DEBY oysters produced in 2014 and 2015 (Table 1). The DEBY15 triploids from the York River were verified by examining a sample of 25 oysters via flow cytometry in June 2015 and were 100% triploid. A sample of 15 oysters was taken from the DEBY14 triploid group in June 2015 and examined for ploidy via flow cytometry and were also 100% triploid. Oysters were shucked and approximately a 4 mm histological section of whole oyster tissue was cut perpendicular to the anterior–posterior axis, just ventral to the labial palps. The *slab* and *corpus* were individually weighed and transferred to a 15 ml falcon tube and a 60 ml polypropylene jar, respectively. The samples were immediately frozen and held at –80°C until further preparation and analyses.

#### Freeze-Drying, Homogenization, and Near Infrared Reflectance Spectra Measurements

All the oyster meats collected were freeze-dried using a Labconco FreeZone 6 or Labconco FreeZone 4.5 (Kansas City, MO) for 72 h. The dried samples were ground to powder using a mortar and pestle and stored at –80°C until spectra acquisition. Between samples, the pestle and mortar were rinsed thoroughly with water and dried. Near infrared spectra were collected using an Analytical Spectral Device LabSpec 5000 spectrometer fitted with an ASD Pro Reflectance probe (ASD, Inc., Boulder, CO). The probe was put in contact with the surface of the oyster freeze-dried powder. Near infrared reflectance spectroscopy irradiations produced reflectance spectra, which were recorded using Indico Pro Software version 5.6

(ASD, Inc., Boulder, CO). For each sample, 3–5 spectra of 50 scans were recorded at different places in the jar or falcon tube and averaged using ViewSpec Pro Software version 6.2 (ASD, Inc., Boulder, CO). From the samples used in the creation of the quantitative NIRS model (calibration set), spectra acquisition was made on the whole freeze-dried oyster meats. For the *slab* experiment, the *slab* and the *corpus* were measured separately.

#### Glycogen Extractions

For samples used to develop a NIRS glycogen model, glycogen extractions were performed using trichloroacetic acid/ethanol procedure, and glycogen measurements were made using a colorimetric iodine method. Both techniques are described in Guévelou and Allen (2016). For samples used in the calibration set, extraction and measurements were performed on the whole freeze-dried oyster meats. Glycogen was not extracted from samples for the *slab* experiment.

#### Creation of Quantitative NIRS Model

For the model calibration, the same procedures as described in Guévelou and Allen (2016) were used. Spectral data analysis and chemometric model development was performed using GRAMS IQ software version 9.1 (Thermo Fisher Scientific, Inc., Waltham, MA), ViewSpec Pro Software version 6.2 (ASD Inc., Boulder, CO), and recommendations of the software user manuals provided by ASD Inc. (Boulder, CO). The data for spectra and associated chemical compositions were loaded into the GRAMS IQ software. All models were developed using coefficient of determination for calibration ( $R^2_{\text{cal}}$ ), standard error of cross validation (SECV), and partial least squares. To build the model, several spectral and mathematical treatments offered by the software were tested. Preprocessing corrections targeting pathlength, multiplicative scatter correction (Isaksson & Næs 1988), and targeting derivatives, Savitzky–Golay (Savitzky & Golay 1964), algorithm methods were used. The selection of spectral regions with the most influence on the models was made using the factor-loading plot provided by the software. Outliers were removed from the calibration set based on examination of the spectral residual plots and regression provided by the software. As suggested by the software user manuals provided by ASD Inc. (Boulder, CO), a threshold of a Mahalanobis distance greater than three was used to confirm outliers. The best calibration model was selected based on the highest  $R^2_{\text{cal}}$  and lowest SECV. The robustness of the calibration models were tested using an independent validation. These samples represent true unknowns that provide validation of the calibration statistics. A total of 20% of oysters used in the calibration set were randomly set aside at the beginning of modeling. The resulting prediction was compared with the empirical data on chemical composition. The  $R^2_{\text{val}}$ , the standard error of prediction, and the RPD were used to test the prediction accuracy of the models.

The calculation formula of  $R^2_{\text{cal}}$  and  $R^2_{\text{val}}$  were as follows:

$$R^2_{\text{cal}}/R^2_{\text{val}} = \frac{\sum_{i=1}^n (Yp_i - \bar{Yk})^2}{\sum_{i=1}^n (Yk_i - \bar{Yk})^2}$$

The calculation formula of SECV and standard error of prediction (SEP) were as follows:



$$\text{SECV/SEP} = \sqrt{\frac{\sum_{i=1}^n (Yk_i - Ykp_i)^2}{n}}$$

The calculation of the RPD were as follows:

$$\text{RPD} = \frac{\text{SD}_{\text{val}}}{\text{SEP}}$$

In all the following equations,  $Yk$  is the known concentration,  $Yp$  is the predicted concentration,  $n$  is the number of samples in the set, and  $\text{SD}_{\text{val}}$  is the standard deviation of the validation set.

#### Comparison of the Glycogen Concentration in the Corpus and Whole Oyster

After calculating the GC (% dry weight) in the *slab* and *corpus* for the select 79 samples, the GC in the whole oyster meat was calculated using the following equation:

$$\text{whole GC} = \frac{(\text{corpus GC})(\text{corpus dry weight}) + (\text{slab GC})(\text{slab dry weight})}{(\text{slab dry weight} + \text{corpus dry weight})}$$

The *corpus* GC and the whole GC were then compared statistically. First, the whole GC was modeled from the *corpus* GC using a simple linear regression. The whole GC data were visually inspected for normality, and the model residuals were visually examined for homogeneity of variance. The whole and *corpus* GC were also compared using a signed-rank test at  $\alpha = 0.05$ , to test the null hypothesis that the median difference between pairs of observations was zero. Statgraphics Software (StatPoint Technologies, Inc.) was used for these comparisons.

#### Determination of Factors Influencing Glycogen Concentration

To determine if shell height, ploidy, or the dry weight ratio of the *corpus*: whole (whole oyster meat) significantly affected GC in the *corpus*, a multiple linear regression (MLR) was used:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \delta_k + \varepsilon_{ijk}$$

where  $Y_{ijk}$  is the GC in the *corpus*,  $\mu$  is the overall mean GC in all *corpus* samples,  $\alpha$  is the effect of shell height,  $\beta$  is the effect of ploidy,  $\delta$  is the effect of the dry weight ratio of *corpus*: whole, and  $\varepsilon_{ijk}$  is the residual error. All sampled oysters were used in the model ( $n = 79$ ). The data for GC in the *corpus* were visually assessed for normality, independent variables were checked for collinearity, and the residuals were visually examined for homogeneity of variance. Significance of the model was determined using an  $F$ -test ( $\alpha = 0.05$ ) and significance of each regression coefficient was determined from the  $t$ -test statistic ( $\alpha = 0.05$ ). The MLR and associated statistical evaluations were done using R 3.0.2 (R Core Team 2013).

## RESULTS

#### Calibration of NIRS Model for Glycogen on Freeze-Dried Samples

To build the freeze-dried NIRS model, a calibration set of 381 samples was used (Table 1). In the calibration set, the GC estimated in freeze-dried tissues was highly variable, from 0%–30.3% of

the dry weight, with a mean  $\pm$  SD of 8.7%  $\pm$  6.7% (Table 2). The best NIRS glycogen model used the wavelength range of 1,154–2,500 nm, multiplicative scatter correction, and first derivative correction Savitzky–Golay at 53 points. This calibration model had an  $R^2_{\text{cal}}$  of 0.96 and an SECV of 1.27% (Table 3). Independent validation showed high  $R^2_{\text{val}}$  ( $= 0.96$ ) and high RPD ( $= 5.2$ ) (Table 3).

#### Relationships between the GC in the Slab, Corpus, and Whole Oyster

##### Correlation between the Glycogen Content in *Corpus* and the Whole Oyster

The glycogen concentration in samples of the *slab* and *corpus* were estimated using the newly developed NIRS calibration model for freeze-dried tissues. To determine if the sample was represented by the NIRS model, the Mahalanobis distance was used. The range and mean of the estimated GC were both slightly higher in the *slab* than in the *corpus* as the *slab* GC ranged from 2.0%–36.7% and the *corpus* GC ranged from 1.4%–31.9%. The mean GC  $\pm$  SD in the *slab* was 16.6%  $\pm$  9.2% and 15.6%  $\pm$  8.5% in the *corpus*. The whole GC ranged from 2.0%–31.7% with a mean and standard deviation of 15.8%  $\pm$  8.4% (Table 2). Differences between paired observations of GC in the *corpus* and whole were not normally distributed, so the signed-rank test was used, and the null hypothesis that the median difference between paired observations was zero was rejected ( $V = 1136$ ,  $P < 0.05$ ). A simple linear regression was used to model the whole GC from the *corpus* GC. The model indicated a strong correlation with an  $R^2$  of 0.99, a slope of one, and an intercept of 0.31 (Fig. 1). From an  $F$ -test, the overall model was determined to be significant ( $F = 5362$ ,  $P < 0.05$ ) and the factor of *corpus* GC was significant ( $t = 73.22$ ,  $P < 0.05$ ). Two outliers were identified in the model, defined as having a studentized residual greater than three (3.6 and 5.1). Notably, both outliers had a much lower ratio of *corpus* GC: whole GC (0.61 and 0.45) than the mean ratio (0.98).

##### *Corpus* GC, Shell Height, and Dry Weight Ratio of the *Corpus* to Whole for Diploids and Triploids

*Corpus* GC, shell height, and dry weight ratio of the *corpus* to the whole oyster meat varied between diploids and triploids

TABLE 2.

Glycogen concentration (% dry weight) measured by iodine method in *Crassostrea virginica* used in calibration of the NIRS model on freeze-dried tissue and estimated glycogen concentration in the *slab*, *corpus*, total oyster meat, and ratio of glycogen concentration in the *corpus* to the total oyster meat (*corpus*: total oyster meat).

Set	$n$	Range	$\bar{x} \pm \text{SD}$
Calibration set	381	0.0–30.3	8.7 $\pm$ 6.7
NIRS predicted glycogen concentration estimation			
<i>Corpus</i>	79	1.4–31.9	15.6 $\pm$ 8.5
<i>Slab</i>	79	2.0–36.7	16.6 $\pm$ 9.2
Total oyster meat	79	2.0–31.7	15.8 $\pm$ 8.4
<i>Corpus</i> : total oyster meat	79	0.5–1.2	0.98 $\pm$ 0.1

Indicated are number of samples ( $n$ ), range, sample mean ( $\bar{x}$ ), and standard deviation (SD).

TABLE 3.

Parameters used to develop models to predict glycogen concentration (% dry weight) and resulting statistics obtained for the calibration set for *Crassostrea virginica* samples.

Chemical parameter	Model parameter			Number of factors (PLS)	Calibration			Independent validation		
	Wavelength range	Pathlength correction	Derivative		$R^2_{cal}$	SECV	Outliers	$R^2_{val}$	SEP	RPD
Glycogen	1,154–2,500	MSC	SG 1 <sup>st</sup> - 53 pts	15	0.96	1.27	1	0.96	1.31	5.2

MSC, multiplicative scatter correction; PLS, partial least squares;  $R^2$ , coefficient of determination; RPD, residual predictive deviation; SECV, standard error of cross validation; SEP, standard error of prediction; SG, Savitzky–Golay smoothing.

used in the *slab* experiment. *Corpus* GC in diploids ranged from 1.4%–20.2% with a mean  $\pm$  SD of 9.1%  $\pm$  4.7%. In triploids, *corpus* GC had a larger range (10.4%  $\pm$  31.9%), mean, and SD (21.9%  $\pm$  6.2%) (Table 4). For shell height, diploids ranged from 58–110 mm, and the mean  $\pm$  SD was 85.1  $\pm$  15.1 mm (Table 4). The triploid shell height had a smaller range of 57–99 mm and a smaller mean  $\pm$  SD of 81  $\pm$  8.4 mm. Oysters were divided into three size classes based on the shell height for comparison of *corpus* GC and shell height (Table 5). The diploids with shell height 58–75 mm had a range of 1.4%–11.7% and a mean  $\pm$  SD of 5.3%  $\pm$  3.2% for *corpus* GC. For triploids with shell height 58–75 mm, the range was 10.4%–23.8% with a mean  $\pm$  SD of 16.1%  $\pm$  5.0%. For *corpus* GC in oysters 76–88 mm in shell height, diploids had a range of 4.0%–19.2% and a mean  $\pm$  SD of 8.6%  $\pm$  4.5% and triploids had a range of 12.2%–31.9% and 23.4%  $\pm$  6.0% for the mean  $\pm$  SD. At a shell height 88–110 mm, the diploids had *corpus* GC ranging from 4.5%–20.2% with a mean  $\pm$  SD of 11.7%  $\pm$  4.0%, and triploids had a range of 17.0%  $\pm$  31.2% with a mean  $\pm$  SD of 24.2%  $\pm$  5.1%. The range

of dry weight ratio of the *corpus* to the whole oyster meat in diploids was 0.67–0.84 g with a mean  $\pm$  SD of 0.79  $\pm$  0.04 g. The results were similar for triploids, with a range of 0.67–0.82 g and mean  $\pm$  SD of 0.78  $\pm$  0.03 g.

#### MLR for Factors Affecting GC in the *Corpus*

A MLR involving shell height, ploidy, and the dry weight ratio of the *corpus* to whole oyster meat was used to explain the *corpus* GC in all sampled oysters ( $n = 79$ ). The distribution of *corpus* GC data was approximately normal, homogeneity of variance was confirmed from examining the residuals, and no collinearity was present. Collectively, the factors had a significant effect on *corpus* GC ( $F = 48$ ,  $P < 0.05$ ). Shell height and ploidy had significant effects on *corpus* GC ( $t = 4.1$ ,  $P < 0.05$ ;  $t = 11.8$ ,  $P < 0.05$ , respectively), whereas the dry weight ratio of *corpus*: whole did not ( $t = -0.04$ ,  $P > 0.05$ ) (Table 6). The estimated coefficients for shell height were 0.002 and 0.136 for triploids, meaning *corpus* GC increased with oyster size and was greater in triploids (Table 6). The model explained 64% of the variation in the *corpus* GC (adjusted  $R^2 = 0.64$ ).

## DISCUSSION

#### Freeze-Dry NIRS Glycogen Model

The main objective of this study was to develop a NIRS model to quantify glycogen in freeze-dried samples of *Crassostrea virginica*. The range of GC encountered using the colorimetric iodine method was representative of the natural range of variation (4.4%–39.2%) reported for *C. virginica* in other studies (Lee & Pepper 1956, Lee et al. 1960, Galtsoff 1964, Sidwell et al. 1979). This range of GC can be explained by the variation inherent among samples used for this glycogen model, which differ in ploidy level, lineage, harvest date, collection site, height, meat weight, and likely gametogenic stage. Compared with previous NIRS models, this freeze-dry model includes the highest number of individual samples (381) built into a model for oysters.

The present study represents the first time a NIRS model has been developed on freeze-dried *Crassostrea virginica* samples. Several NIRS models that use homogenized tissues of whole oysters to measure biochemicals, notably glycogen and lipids, have been developed on different species of oysters, for example, *Crassostrea gigas* (Brown 2011, Brown et al. 2012, Madigan et al. 2013), *Saccostrea glomerata* (Brown et al. 2012), and *C. virginica* (Guévelou & Allen 2016). Only one study reports NIRS models developed on freeze-dried oysters, that being for *C. gigas* (Wang et al. 2015). Wang et al. (2015)

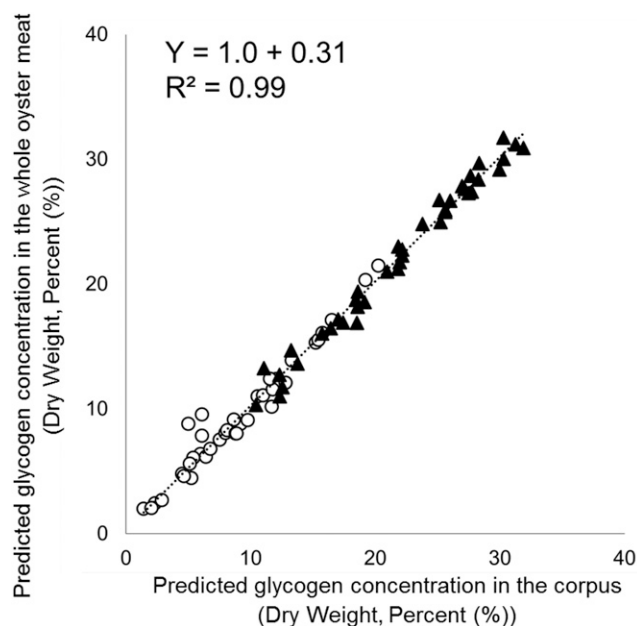


Figure 1. Linear regression comparison between estimated glycogen concentration (% dry weight) measured by NIRS in the *corpus* and in the whole oyster meat for *Crassostrea virginica* sampled in the York River and Rappahannock River, VA, in August 2016 ( $n = 79$ ). White circles represent data for diploids and black triangles for triploids.

TABLE 4.

Glycogen concentration (% dry weight) in the *corpus*, shell height, and ratio of *corpus* dry weight to whole meat dry weight for diploids, triploids, and all *Crassostrea virginica* samples sampled in the York River and Rappahannock River, VA, in August 2016 ( $n = 79$ ).

	Corpus GC (%)		Shell height (mm)		C:W	
	Range	$\bar{x} \pm SD$	Range	$\bar{x} \pm SD$	Range	$\bar{x} \pm SD$
Diploid	1.4–20.2	9.1 $\pm$ 4.7	58–110	85.1 $\pm$ 15.1	0.67–0.84	0.79 $\pm$ 0.04
Triploid	10.4–31.9	21.9 $\pm$ 6.2	57–99	81 $\pm$ 8.4	0.67–0.82	0.78 $\pm$ 0.03
All oysters	1.4–31.9	15.5 $\pm$ 8.5	57–110	83.0 $\pm$ 12.3	0.67–0.84	0.78 $\pm$ 0.03

GC, glycogen concentration; C, *corpus*; W, whole oyster meat;  $\bar{x}$ , sample mean; SD, standard deviation.

developed models to quantify glycogen, protein, fat, taurine, zinc, selenium, and ash in the gonad-visceral mass and adductor muscle. The glycogen model reached an  $R^2_{\text{val}}$  of 0.99, an SECV of 1.71%, and an RPD of 6.70 (Wang et al. 2015). In the present study, the glycogen model was developed on the soft tissue of the entire oyster and reached an  $R^2_{\text{val}}$  of 0.96 and an RPD of 5.2. These parameters confirmed the robustness of this model. The model is considered suitable for quantification if the  $R^2$  values are greater than 0.90 and if the values from calibration ( $R^2_{\text{cal}}$ ) and validation ( $R^2_{\text{val}}$ ) are similar (Urbano-Cuadrado et al. 2004). The RPD value is also a criterion of robustness where a value greater than 3.0 indicates the model is adequate for analytical purposes for agricultural products (Williams & Norris 2001). Although there are several discrepancies in the material and methods between the two studies, both reached high values of  $R^2_{\text{val}}$  and RPD. The main differences between these two studies are that Wang et al. (2015) used only part of the oyster (gonad-visceral mass and/or adductor muscle) and also sieved the ground, freeze-dried powder on a 177  $\mu\text{m}$  screen. Although the reason behind the sieving is not completely explained, it can be assumed the intention was to make the powder more homogenous, and therefore may explain the higher  $R^2_{\text{val}}$  and RPD observed in their glycogen model. In this study, the main difficulty occurring during the homogenization process was the grinding of the adductor muscle. The adductor muscle plays a crucial role of controlling the valves and is composed of two parts: a translucent, larger section and a smaller, white, crescent-shaped part (Eble & Scro 1996). The muscle bands of the white part have been described to be more compact and are surrounded by tougher connective tissues than those of the translucent part (Galtsoff 1964). Even after several minutes of grinding using mortar and

pestle, in some samples, relatively large pieces (>1 mm<sup>2</sup>) of the white muscle were found in the powder. The presence of relatively large pieces of the adductor muscle in the powder may explain a certain imprecision in the glycogen estimation using NIRS.

The first NIRS models developed on *Crassostrea virginica* oysters included both moisture and glycogen models (Guévelou & Allen 2016). The two models were developed from oyster slurries of homogenized whole oyster meats, and the glycogen model had an  $R^2_{\text{cal}}$  of 0.94, an SECV of 0.36%, and an of 4.1. The freeze-dry glycogen NIRS model in the present study is an improved version of the model in Guévelou and Allen (2016), as all of the samples used in Guévelou and Allen (2016) were used for the present NIRS freeze-dry glycogen model. Therefore, the freeze-dry model includes 138 samples used to build the previous model, as well as 243 additional samples. The result was a higher  $R^2_{\text{cal}}$  value (0.96), SECV (1.27%) and RPD (5.2).

To build a NIRS quantitative is an active process where the developer goes through successive iterations from outlier removal, choice of processing option, to region selection. Interestingly, in this freeze-dried model, there was only one outlier from the calibration set, whereas 12 outliers were identified when the first model was developed. With more samples, this new model presents a larger range of GC values, improving its robustness. In this model, a broader wavelength selection appears to give better experiment statistics (1,154–2,500 nm). In comparison, Guévelou and Allen (2016) used a slighter wavelength selection (1,380–2,095 nm) to develop a quantitative glycogen model on fresh *Crassostrea virginica* tissues. Alternatively, Wang et al. (2015), claims to have a better glycogen model on freeze-dried *Crassostrea gigas* combining three distinctive wavelength regions 1,204–1,283; 1,768–1,936; and 1,978–2,167 nm. In addition, freeze-drying the

TABLE 5.

Glycogen concentration (% dry weight) in the *corpus* for diploid, triploid, and *Crassostrea virginica* sampled in the York River and Rappahannock River, VA, in August 2016 ( $n = 79$ ) based on shell height.

	Shell height 58–75 mm			Shell height 76–88 mm			Shell height 88–110 mm		
	$n$	Range	$\bar{x} \pm SD$	$n$	Range	$\bar{x} \pm SD$	$n$	Range	$\bar{x} \pm SD$
Diploid	11	1.4–11.7	5.3 $\pm$ 3.2	10	4.0–19.2	8.6 $\pm$ 4.5	18	4.5–20.2	11.7 $\pm$ 4.0
Triploid	8	10.4–23.8	16.1 $\pm$ 5.0	23	12.2–31.9	23.4 $\pm$ 6.0	6	17.0–31.2	24.2 $\pm$ 5.1
All oysters	19	1.4–23.8	9.9 $\pm$ 6.8	33	4.0–31.9	18.9 $\pm$ 8.8	24	4.5–31.2	14.8 $\pm$ 7.0

X, sample mean; SD, standard deviation.

TABLE 6.

Factors, coefficient estimates, standard error (SE), *t*-test statistic (*t*), and *P* value (*P*) for the intercept and independent variables used in a multiple linear regression to model *corpus* glycogen concentration in *Crassostrea virginica* sampled in the York River and Rappahannock River, VA, in August 2016 ( $n = 79$ ).

Factors	Coefficient estimates	SE	<i>t</i>	<i>P</i>
Intercept	-0.069	0.008	-8.445	<0.05
Shell length	0.002	0.001	4.055	<0.05
Ploidy (triploid)	0.136	0.012	11.784	<0.05
<i>Corpus</i> : Whole	0.006	0.189	-0.035	>0.05

samples likely increased the accuracy of the measurements. Water is an important constituent of oysters, often making up about 80% of oyster wet weight (Lee & Pepper 1956, Lee et al. 1960, Galtsoff 1964, Sidwell et al. 1979), and removal of this moisture allows the operator to have better control over biochemical variation in the concentration and temperature fluctuation. Generally, it was easier to generate spectra using freeze-dry powder compared with wet samples.

#### Case Study on Glycogen Concentration in Two Different Sections of the Oyster

The second part of this study used the current model on freeze-dried oysters to determine GC in two different sections of an oyster: the histological cross-section usually used for histological analysis (*slab*) and the oyster meat remaining after sampling (*corpus*). A cross-section of the visceral mass is often dissected for health diagnostics and to examine the gametogenic stage of the oyster, and often the *corpus* is analyzed and used as a proxy for the whole oyster meat (e.g., Arcos et al. 2009, Guévelou et al. 2013, Jeung et al. 2016). Although a common proxy, the question remained, how accurate is the estimation of GC in the *corpus* as a proxy for GC in the tissues of the whole oyster? Given GC varies among different organs within the oyster, it seemed possible that the *slab* GC and *corpus* GC could be different, and thus the *corpus* and whole oyster could have different GC. Berthelin et al. (2000) described the variation in glycogen within *Crassostrea gigas*, as glycogen storage cells were found mainly in the labial palps, mantle, and gonadal area, with lesser numbers of cells in the gills and digestive area.

In the present study, a strong correlation ( $R^2 = 0.99$ ) was found between the GC in the whole oyster meat and in the *corpus*, however, the median difference was not zero, as determined by a signed-rank test. The difference suggests that measuring the *corpus* slightly underestimates the GC in the entire oyster, as the mean and median GC of the *corpus* was less than that of the whole oyster meat (means: 15.6% and 15.8%, respectively; medians: 13.7 and 14.7, respectively). Despite the statistical difference, the magnitude of the difference is small and the correlation between the GC in the whole oyster and *corpus* is strong ( $R^2 = 0.99$ ) so that the *corpus* appears as an accurate proxy for the GC in the whole oyster meat.

The second aspect of the present investigation in using the *corpus* as a proxy for the entire oyster meat was to determine what factors could influence the GC of the *corpus*. One consideration was that the GC of the *corpus* could be

significantly affected by the sampling process. For instance, the relative ratio of the size of the *corpus* to the whole oyster meat (0.67–0.84 in dry weight) differs with the overall size of the oyster. Given these differences between the *slab* and the *corpus* were observed. For one, the GC was higher in the *slab* than in the *corpus* ( $16.6\% \pm 9.2\%$  and  $15.6\% \pm 8.5\%$ , respectively). Also, a higher Mahalanobis estimation was observed in the *slab* in comparison with the *corpus* (2.9 and 1.6, respectively), suggesting the *slab* was not as well represented by the glycogen NIRS model built on whole oyster samples. Both differences suggest the *slab* composition is slightly different than that of the *corpus*, and therefore the relative size of the *slab* could significantly affect the estimation of GC in the *corpus*. Other considerations were that the ploidy and the total size of the oyster could affect GC. From a MLR, no significant effect from the size ratio in the *corpus* to the whole oyster meat on GC was found, but GC in the *corpus* was significantly affected by both ploidy and the height of the oyster.

In both diploid and triploid oysters, the *corpus* GC increased with the height of the animal, suggesting that bigger oysters have higher GC. Size has been found to affect GC in several bivalves where smaller, younger adults contain significantly lower GC than larger, older adults (for *Ostrea edulis* see Holland & Hannant 1974, 1976, for *Chlamys islandica* see Sundet & Vahl 1981, and for *Cerastoderma edule* see Navarro et al. 1989, Newell & Bayne 1980). The difference in GC between small and large animals may be partly explained by the relatively higher weight-specific metabolic demand in smaller oysters (Dame 1972, Thompson & Bayne 1974). Higher weight-specific metabolic demand could reduce the ability of an oyster to build and maintain glycogen reserves as more glycogen is required for metabolic needs.

Despite previous findings that size affects GC, many prior studies have measured GC in oysters to assess condition or reproductive status (e.g., Perdue et al. 1981, Allen & Downing 1986) without taking into account the possible bias associated with using oysters of different sizes. Although it is logical that faster growing, larger oysters may be in relatively better condition than smaller oysters, a size metric such as shell height does not in itself dictate a health status. Size does, however, appear to affect weight-specific metabolic demand, and therefore a GC in a small oyster may indicate a different health status than the same GC in a larger oyster. Size may, therefore, be a confounding variable in studies comparing GC among oysters. In the future, a size metric, such as shell height, should be considered as a covariate in studies involving glycogen measurements, even when glycogen is reported in percent, as was done in the current study.

From the 39 diploid and 40 triploid oysters sampled in this study, triploids had a significantly higher mean concentration of glycogen. In conditions conducive to gametogenesis, higher concentrations of carbohydrates have been found in triploid *Crassostrea gigas* compared with diploid counterparts, which has been linked to the reduced fecundity in triploid oysters (Allen & Downing 1986, Shpigel et al. 1992). The relationship between the storage and utilization of carbohydrates, which in oysters is primarily glycogen, and gametogenesis in oysters is well established, as diploid oysters expend stored glycogen reserves during gametogenesis. In diploid *Crassostrea virginica*, glycogen content has generally been observed to reach a maximum before the onset of gametogenesis and fall to a minimum immediately after spawning (Chipman 1947, Engle 1951, Barber et al. 1988). The maximum and minimum can, therefore, occur



within as little as three months in diploids (Allen & Downing 1986). Allen and Downing (1986) observed a much different pattern of glycogen utilization in triploids, as glycogen content in triploids decreased more steadily and remained higher than in diploids over the spring and summer. Diploids and triploids were sampled in August for this study, which is within the spawning window for oysters in the lower part of the Chesapeake Bay (USA) and may explain the higher concentration of glycogen in sampled triploids.

The first applications of the NIRS model to compare GC in freeze-dried *Crassostrea virginica* samples had some limitations. For one, oysters were only sampled at one time period in August of 2016. Owing to changes in GCs seasonally, oysters sampled in the fall, winter, or spring will certainly vary. In this study, the majority of the 39 sampled diploid oysters had little visible gonadal development (no reproductive data were collected). The results may be especially different if oysters were sampled earlier in the year while expending significant gametogenic effort. Extensive gonadal development may concentrate high levels of glycogen, lipids, and proteins to the viscera in the form of gametes, increasing the difference in the biochemical composition of the *slab* and the *corpus*. The diploid outliers identified in this study, which had a much lower ratio of GC in the *corpus* as compared with the entire oyster (0.45 and 0.61 versus the mean of 0.98), could possibly be from advanced gonadal development.

In this study, a NIRS model to measure glycogen in freeze-dried *Crassostrea virginica* oyster tissues was successfully developed and applied to determine variation in GC within and among oysters. The strong ( $R^2_{\text{val}} = 0.96$ ) correlation between

the concentration of glycogen measured via NIRS and traditional methods indicates that this model provides a useful alternative to costly and time consuming traditional laboratory-based methods. More broadly, NIRS technology has potential of multicomponent analysis. That is, once models have been built, future, current, and older spectra can be used to qualify or quantify new parameters. From the application of this new model, a strong correlation in GC in the *corpus* and the whole oyster meat was determined, and the average GC in the *corpus* was nearly the same as that in the whole oyster meat. After determining the *corpus* as an effective proxy for the whole oyster meat, the *corpus* GC was used to determine a significant positive relationship between shell height and GC and that ploidy had a significant effect on GC.

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