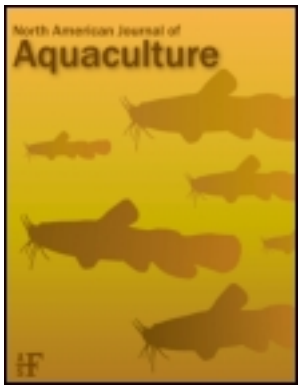

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Rapid Estimation of Gonad-to-Body Ratio in Eastern Oysters by Image Analysis

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TECHNICAL NOTE

Rapid Estimation of Gonad-to-Body Ratio in Eastern Oysters by Image Analysis

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Abstract

The goal of this study was to develop a rapid and reliable method of quantifying the gonadal condition of eastern oysters *Crassostrea virginica* based on a gonad-to-body ratio (GBR). The objectives were to compare a previously established transect method with three alternative computer-based image analysis (IA) methods based on acquisition time, composite GBR values, and GBR values at different stages of gonadal development. The first IA method calculated the area of the gonad and body while eliminating the dorsal and ventral curvature of the gonad, the second calculated the area of the gonad and body while eliminating the gill area, and the third calculated the total area of the gonad and body. The GBR values from the first and third IA methods were not different from those from the transect method. The second IA method resulted in higher GBR values in 80% of the measurements compared with the other three methods. However, measuring the gonadal area and total body area was five times faster than with the transect method and, excluding the curvature, was nine times faster but susceptible to visual error. Based on the speed and comparability with the transect method, the third IA method was the most useful for quantifying gonadal development.

The Food and Agricultural Organization of the United Nations valued the global oyster yield produced by aquaculture at almost US$3.2 billion in 2008 (FIPS 2010). Although large, global and local oyster production can be variable and is vulnerable to significant losses due to disease or poor recruitment (Supan 2000). Methods to assess gonadal condition of oyster broodstocks are important to maintain a dependable supply of larvae for culture. Although histological sectioning is expensive and time consuming, and therefore not a practical tool for real-time hatchery evaluation of broodstock (Supan and Wilson 2001), its use is essential in experimental studies that evaluate and characterize gonadal development.

Several methods have been used to quantify gonadal condition in oysters and are commonly performed by means of transverse histological sections through the entire oyster body (Supan and Wilson 2001). Some of these methods measure only gonadal and body widths in a predetermined number of transects (Kennedy and Battle 1964), while others determine the gonadal and body areas by planimetry (Morales-Alamo and Mann 1989) or by computer-based image analysis (IA) of specific areas of

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the gonad (Heffernan and Walker 1989). The abundance of different methods and a lack of standardization make it difficult or impossible to compare published results; this raises concerns that some of the apparent differences in gonadal estimations are due to methodological differences.

Image analysis has been used to quantify gonadal development in oysters and other bivalves in previous studies with demonstrated reliability (Heffernan and Walker 1989; Buchanan 2001; Delgado and Perez-Camacho 2003). However, these methods only examine random portions of the gonad. An IA method that measures the entire gonadal area could provide more consistent and accurate results. The novelty and utility of the IA methods is the use of digital morphometric analysis based on simple and inexpensive computer software that provides fast, easy, and reliable quantitative methods for comparison.

The goal of this work was to develop a rapid and reliable method to quantify the gonadal condition of eastern oysters *Crassostrea virginica* based on a gonad-to-body ratio (GBR) that would be comparable with the methods currently employed. The objectives of this study were to compare a standard transect method used for this species (Supan and Wilson 2001) with three computer-based IA methods based on (1) acquisition time, (2) composite GBR values, and (3) GBR values at different stages of gonadal development. The three new methods calculated areas based on different geometries of image capture regions and offered benefits compared with the transect method.

METHODS

The eastern oysters used in this study ($n = 50$) were produced at the Sea Grant Grand Isle Bivalve Hatchery (29°15′12″N, 90°03′26″W) on Caminada Bay, Louisiana, in June 2002. Individuals were randomly collected from natural waters late in the normal spawning season (August 2003). All work was performed by laboratory personnel with at least 3 years of undergraduate education and basic training in microscopy and image analysis.

Histology.—The oysters were sectioned for histological comparison according to the methods of Morales-Alamo and Mann (1989) and Howard et al. (2004). In brief, a 4-mm-thick transverse section posterior to the gill–palp junction was removed (Figure 1), placed posterior side up into a tissue cassette (Omnisette; Thermo Fisher Scientific, Waltham, Massachusetts), and preserved in Davidson's fixative (Humason 1967). The tissues were processed (Tissue-Tek VIP 5 vacuum infiltration processor; Sakura Finetek USA, Torrance, California) by serial dehydration in ethanol (70% for 60 min, 80% for 30 min, 95% for 75 min, 100% for 45 min) (Thermo Fisher Scientific) followed by clearing with xylene (Thermo Fisher Scientific) and embedding in paraffin (Paraplast; Thermo Fisher Scientific). Each block was sliced by means of a microtome (Finesse ME+; Thermo Fisher Scientific) into 4-μm-thick sections approximately 1 mm from the gill–palp junction. The histological sections were mounted on microscope slides and

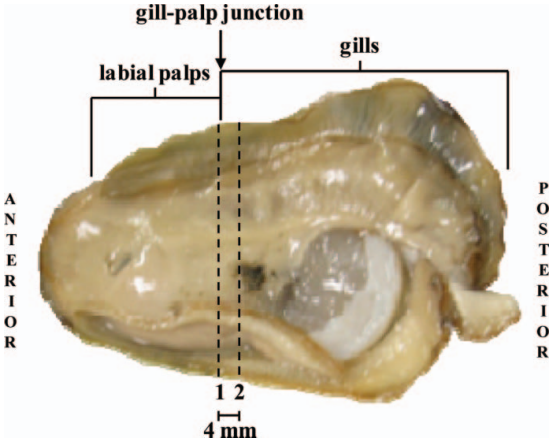

FIGURE 1. Eastern oyster body removed from the shell. A transverse section was collected (between the dashed lines) by performing two cuts with a razor blade, the first at the gill–palp junction and the second 4 mm posterior to the first cut. [Figure available in color online.]

stained (AutoStainer XL; Leica Microsystems, Bannockburn, Illinois). Sections were cleared with xylene for 15 min, dehydrated in absolute ethanol (100%) for 4 min, and washed in tap water for 1 min before they were stained with hematoxylin (Anatech, Battle Creek, Michigan) for 2.5 min and rinsed with tap water for 1 min. The samples were decolorized in acid alcohol (0.5% hydrochloric acid in 70% ethanol) (Thermo Fisher Scientific) for 30 s, washed in tap water for 1 min, blued in ammonia water (0.25% of 58% ammonium hydroxide in tap water) (Thermo Fisher Scientific) for 30 s, rinsed in tap water for 1 min and 95% ethanol for 1 min, and counterstained with eosin (Anatech) for 1 min followed by 2.25 min in absolute ethanol and 3 min in xylene. A cover glass was applied to each slide with Permount mounting media (Thermo Fisher Scientific).

Gonadal staging.—The stained histological sections were evaluated by light microscopy to determine the stage of gonadal development based on the gametogenic classification described by Kennedy and Krantz (1982). Oysters were classified into four stages: early development (ED), late development (LD), spawning (S), and advanced spawning and regressing (ASR). The reproductive state of the gonad was used to examine its effect on each method used to determine the GBR.

Transect method.—The GBR was calculated following a routine methodology (Kennedy and Battle 1964) that used 10 equidistant transects across each section, excluding the middorsal and midventral regions of the gonad, which would determine the gonadal width relative to the body width on each transect. The average GBR value from transects 3–8 was used to determine the total GBR of each oyster as recommended by Supan and Wilson (2001) (Figure 2A). This method is based on sampling across the histological section to determine the ratio of six replicate linear measurements for the gonad and body and was developed for use with a light microscope and an ocular micrometer.

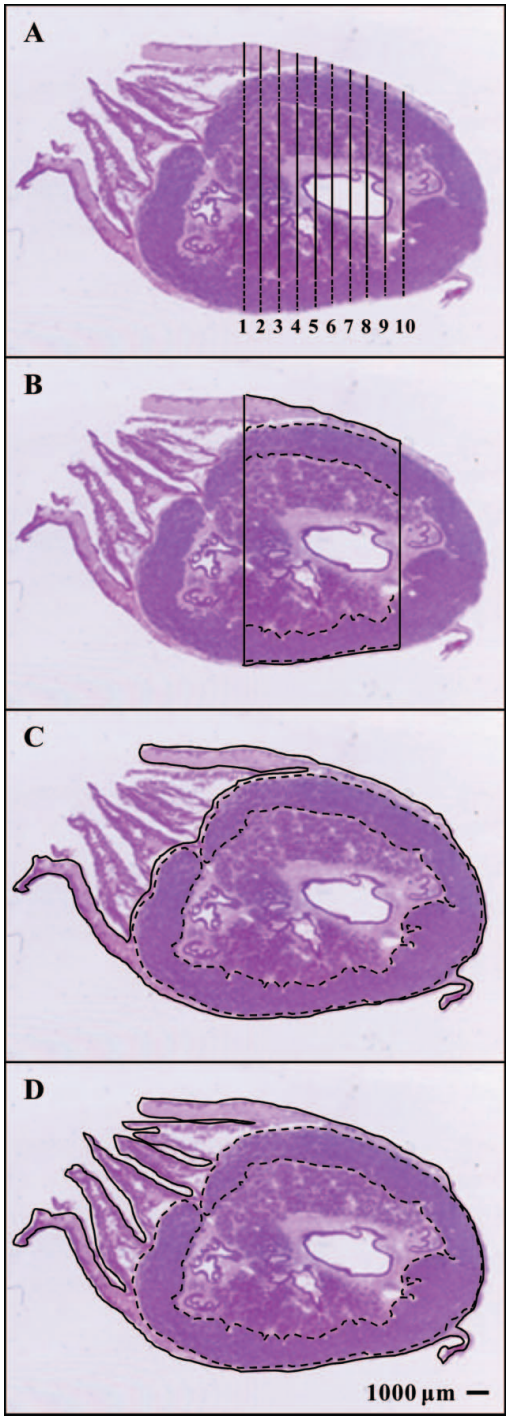

FIGURE 2. Diagrammatic representation of the four methods used to histologically determine the gonad-to-body ratio (GBR) of an eastern oyster. Panel **(A)** depicts the transect method, a standard method of determining the GBR. Measurements from transect numbers 3–8 were used; each line represents one transect, and the dashed portion of each transect demarks the gonadal region. Three image analysis methods were also used and the results compared with those of the transect method: **(B)** exclusion of the gonadal curvature, **(C)** exclusion of the gills from the total body measurement, and **(D)** measurement of the total body area; in each image, the solid lines outline the body area and dashed lines outline the gonadal area. [Figure available in color online.]

Image analysis.—For all three IA methods, the same histological section of each oyster used previously for the transect method was digitized on a flatbed scanner (Epson Perfection 1640SU; Epson America, Long Beach, California) at a resolution of 800 dots per inch (dpi), and the image was imported into Photoshop version 7.0 (Adobe, San Jose, California). The scanned images were magnified 12 times and analyzed with IA software (Metaview 6.1; Universal Imaging, Downingtown, Pennsylvania). The outline of the body (following the outer margin of the mantle) was traced using a digital drawing tablet and stylus (Hyper Pen 12000U; Aiptek, Irvine, California) connected to a personal computer (Dell Precision Workstation 360; Dell, Austin, Texas). The gonad was also traced by following the inner and outer margins and avoiding the interfollicular space to the extent allowable by the magnification of the digital image. The IA software automatically determined the areas of the body and the gonad by counting the number of pixels contained within the region traced. The pixel number within the gonadal trace was divided by the pixel number within the trace of the body to calculate the GBR.

The first IA method calculated the GBR by comparing the area of the gonad with the area of the body only in a section of the slide similar to the section used in the transect method by excluding the curvature of the middorsal and midventral regions of the gonad (Figure 2B). The second IA method calculated the GBR by comparing the total area of the gonad with the area of the body excluding the gills (Figure 2C). The third IA method calculated the GBR by comparing the total area of the gonad with the total area of the body including the gills (Figure 2D).

The time spent acquiring the area measurements to calculate the GBR by each method was recorded and compared to determine the effort required for each method.

Statistical analysis.—All analyses were performed with SAS statistical software (SAS version 9.1.3, SAS Institute, Cary, North Carolina). The GBR values obtained by each method were analyzed and compared by means of the Friedman test (Zar 1974); Dunn's posthoc procedure was used to test for differences among the four methods. An analysis of variance (ANOVA) followed by a Tukey's post hoc test was performed to test for differences in the time required for each method. A significance level of $\alpha = 0.05$ was used in all statistical analyses.

RESULTS

All four stages of development were present in this study; the spawning stage (S) was the most prevalent in the oysters sampled (Table 1). The prominent characteristic used to delineate the gonad was the dark basophilic staining of its densely packed gametes. The total body area was defined as all tissues in each histological section including the gonad. As stated above, the area measured to calculate the GBR was defined for each method used in this study.

The time to acquire the GBR was significantly different ($P < 0.05$) among the transect method and the three IA methods.

TABLE 1. Statistical comparisons of the four methods used to determine the gonad-to-body ratio (GBR) in eastern oysters. Values are means ± SEs; within columns, different lowercase letters indicate significant differences ($P < 0.05$) among the transect method and three image-analysis-based methods as determined by Tukey's test (acquisition time) and Dunn's test (GBR).

			GBR for each gonadal stage			
Method	Acquisition time (min)	Composite GBR	Early development ($n = 5$)	Late development ($n = 32$)	Spawning ($n = 6$)	Advanced spawning and regression ($n = 7$)
Transect	14.7 ± 0.3 z	0.213 ± 0.011 y	0.082 ± 0.015 z	0.174 ± 0.022 zy	0.251 ± 0.011 y	0.148 ± 0.015 y
IA, no curvature	1.6 ± 0.1 x	0.214 ± 0.012 y	0.069 ± 0.005 z	0.168 ± 0.022 y	0.258 ± 0.011 y	0.158 ± 0.012 zy
IA, no gills	2.7 ± 0.1 y	0.258 ± 0.013 z	0.089 ± 0.007 z	0.203 ± 0.014 z	0.309 ± 0.013 z	0.186 ± 0.016 z
IA, total body area	3.1 ± 0.2 y	0.237 ± 0.013 y	0.072 ± 0.010 z	0.178 ± 0.016 y	0.289 ± 0.012 y	0.162 ± 0.013 zy

Compared to the transect method, the average time for IA was 5 times faster when tracing the total body area and gonad, and 10 times faster when tracing excluded the curvature; there was no significant difference in time taken between measuring the total body area and body area excluding the gills (Table 1).

There were no significant differences ($P > 0.05$) among the composite (total) GBR values derived from two of the IA methods tested (the first excluding the curvature and the third measuring the total body area) and the transect method (Table 1). The differences among the GBR values obtained for any oyster by these three methods were never greater than 10%. The GBR calculated when the gills were excluded during measurement was significantly different ($P < 0.05$) from the other three methods; the estimate of GBR values was higher 80% of the time. These values were always at least 10% greater than the values derived from the other methods.

When the oysters were classified based on their gonadal stage, there were no significant differences ($P > 0.05$) among the four methods when the GBR values were small (<0.09) and the oysters were in either early development or advanced spawning and gonadal regression. When the oysters were in late development or spawning and the GBR values were high (>0.14), there were significant differences among the methods (Table 1) and the results were similar to the composite GBR values in which gonadal stage was not accounted for.

DISCUSSION

The goal of this study was to develop a rapid and reliable method to quantitatively estimate gonadal condition in oysters based on a gonad-to-body ratio. To achieve this, three methods were evaluated to calculate the area of the gonad and body on histological sections with computer-based image analysis. These IA methods were compared with a reference transect method that has been used in published studies to report gonadal condition. Of the IA methods tested, the first, which excluded the curvature of the middorsal and midventral regions of the gonad, and the third, which measured the total body area, were the fastest and most reliable compared with the transect method. Although these two methods yielded results similar to those from the transect method, the exclusion of the gonadal curvatures was susceptible to visual error when the area to be measured was selected and outlined, which could potentially result in unnecessary variation when performed by different technicians.

There were no significant differences among the four methods to estimate GBR values when the ratios were small (during ED). This could be because either there was truly no difference among the methods, or the differences among the GBR values were too small to resolve (<0.02); alternatively, because there were few oysters in the ED stage ($n = 5$), the statistical power could have been insufficient to detect differences among the methods. When GBR values were high (during LD, S, and ASR), significant differences among methods were evident. In the LD, S, and ASR stages, the transect method and the IA methods that (1) excluded the gonadal curvature and (2) measured the total body area were comparable, while the IA method that excluded the gills from the measurement of body area varied in its similarity to the other methods depending on the gonadal stage. Similar to the ED stage, there were only a few oysters in LD ($n = 7$) and ASR ($n = 6$), which decreased the statistical power of the test; the frequency of the S stage was greater and was present in 64% of the oysters ($n = 32$) in this study. In any event, this population of oysters was selected for study because the frequency of stages sampled represented the frequencies observed normally during this stage of the spawning season and represented a conservative test of the methods.

There were several advantages to the computer-based IA methods tested in this work. There was a significant reduction in the time needed to determine the GBR; these methods were less labor intensive, they required minimal knowledge of computer software, and the GBR values were not different from those found with the transect method, thereby allowing direct comparison of results among previous and future studies. Another important difference between the IA methods tested in this study compared with those in other studies is that the total area of the histological section was used to estimate the GBR instead of a subsample of linear fields within the section. Measuring the entire gonadal and body areas reduced the possibility of

erroneous estimation due to sampling error or asymmetrical development of the gonad. Additional data should be collected to increase statistical power of this test and fully validate the results of this study by balancing the sample size in each gonadal stage.

In conclusion, the measurement of the total body area was the most efficient and accurate method and is therefore recommended based on this study. The acquisition time was rapid (an average of 3 min per oyster), which is comparable with existing methods, and was not subject to visual errors by the operator after the histological characteristics of the body and gonad were established.

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