Available online at www.sciencedirect.com
ScienceDirect
Fisheries Research 89 (2008) 84–89
FISHERIES RESEARCH
www.elsevier.com/locate/fishres

Procedures for efficiently producing high-quality fecundity data on a small budget

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Received 8 December 2006; received in revised form 30 August 2007; accepted 14 September 2007

Abstract

Though fecundity estimation has typically been very time consuming, efficient methods have recently been developed. Building on these developments, we describe procedures for efficiently producing precise, accurate, fecundity estimates for highly fecund Atlantic cod, using a low-cost laboratory setup. Capturing images with a standard flatbed scanner and analyzing them with free image analysis software (total equipment cost ≈ $100) we were able to process individual samples in as little as 5 min. We show a strong relationship between mean oocyte diameter (range 200–900 μm) and oocyte density ($r^2 = 0.971$, $n = 50$), and thus are able to estimate fecundity using gravimetric and auto-diametric methods. Testing the precision of the auto-diametric method we find estimates produced by both methods to be highly correlated ($r^2 = 0.961$, $n = 26$). The success of these methods should assure fisheries scientists that fecundity estimation is no longer the daunting task it once was, and catalyze further production of fecundity data.

Keywords: Fecundity; Oocyte size; Digital image analysis; Cod; Auto-diametric method

1. Introduction

The relative lack of fecundity data for cod and other fishes is largely due to the complexity and time consuming nature of methods typically used to count the tiny oocytes of highly fecund, pelagic spawners. Though rapid methods of enumerating oocytes using particle-counters have existed for over 20 years (Witthames and Walker, 1987) they have not become popular, perhaps due to the cumbersome equipment required. The gravimetric method (Kjesbu and Holm, 1994) remains the most common method of fecundity estimation, whereby oocytes in a pre-weighed sub-sample are counted manually, often with a stereomicroscope, and then the result is multiplied by the weight of the entire ovary. But newer, more efficient techniques involving digital imaging are now available, and have advantages over techniques which rely solely on human vision (Friedland et al., 2005; Thorsen and Kjesbu, 2001).

In capturing a digital image of a group of objects, digitizing equipment (e.g. a digital camera) sees the objects quantitatively as an array of independent light measurements which can then be recorded without error onto a computer. Though the human eye may view this same group of objects very clearly, it does not have the same ability to record this information accurately. So while oocytes viewed with the human eye must be immediately analyzed (e.g. counted or measured) by the viewer, the same oocytes captured in a digital image can be viewed and analyzed at another time by a computer, using a variety of image enhancement and analysis procedures found in any of several computer programs (e.g. ImageJ, ImagePro, NIH Image, Optimas, Scion Image). When dealing with animal tissues which are time and environment sensitive, stored images are particularly valuable since they can be easily referenced at later stages of research, and can be reanalyzed without additional lab work or specimen handling.

Digital image analysis has only recently come into use in the study of fish fecundity and oocyte size but has experienced widespread use in the biological sciences for over a decade. Techniques for measuring and counting cells and other small particles have been studied extensively and are explained in detail by Russ (1999).

Two groups of researchers have now developed procedures to rapidly count and measure fish oocytes using digital image analysis, both of which are effective but differ in important ways. Friedland et al. (2005) used a flatbed scanner to capture images

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doi:10.1016/j.fishres.2007.09.005

of the oocytes of developing American shad (*Alosa sapidissima*) then analyzed them used version 6.5 of the Optimas image process system. This analysis produced counts of oocytes within a specified size range that could then be used to estimate potential fecundity using the gravimetric method. In contrast to Friedland et al., Thorsen and Kjesbu (2001) used a video camera connected to a stereomicroscope to capture images of the oocytes of developing Atlantic cod (*Gadus morhua*) then analyzed them using the freeware program NIH-image version 1.62. Their analysis also produced oocyte counts and measurements which were used to estimate fecundity using the gravimetric method, but were then used to develop an entirely new method which they termed the 'auto-diametric' method.

Within the ovary of developing Atlantic cod, vitellogenic oocytes are packed together in a very uniform way. Thorsen and Kjesbu (2001) assessed the uniformity of this packing using digital image analysis to measure and count the oocytes of preweighed sub-samples of ovarian material and discovered that the relationship between mean oocyte diameter and oocyte density was very strong. The function they developed between oocyte diameter (O_{Diameter}) and oocyte density (O_{Density}) is reprinted here as Eq. (1):

$$O_{\text{Density}} = 2.139 \times 10^{11} O_{\text{Diameter}}^{-2.700} (\mu\text{m}) \quad (1)$$

$n = 47$, $r^2 = 0.988$, $300 \leq O_{\text{Diameter}} \leq 850\ \mu\text{m}$.

The high coefficient of determination for the regression illustrates the strength of the relationship. Using this equation to estimate oocyte density from measurements of mean oocyte diameter they then multiplied oocyte density by whole ovary weight, as in the gravimetric method, to estimate fecundity. By producing fecundity estimates with similar precision and accuracy to the gravimetric and sphere-volume methods, they were able to further validate the auto-diametric method.

Since Thorsen and Kjesbu (2001) published their method, it has been evaluated and accepted as valid (Murua et al., 2003) especially for determining fecundity in species with group-synchronous ovaries. It has also been used in at least one published study (Yoneda and Wright, 2004) to estimate fecundity of Atlantic cod. When the auto-diametric method was evaluated by Friedland et al. (2005) for shad they found the relationship between oocyte size and oocyte density to be imprecise, but suggested that this imprecision may be due to preservation technique. In our study, we used 10% phosphate-buffered formalin to preserve and fix ovarian material following Thorsen and Kjesbu (2001), rather than modified Gilson's solution used by Friedland et al. (2005) in order to increase precision.

An advantage of the auto-dianetric method, particularly valuable to researchers collecting ovary samples at sea, is that one needs only measure mean oocyte diameter to be able to estimate oocyte density once the mathematical relationship between mean oocyte diameter and oocyte density is determined. Therefore, precise weighing of small sub-samples of ovarian material, which is very difficult to accomplish on a moving ship, need only be done for the first 50 or so samples collected.

In the present research, we sought to develop a procedure to estimate fecundity of Atlantic cod that was simple and rapid, using an inexpensive set up. Drawing upon the work of Thorsen and Kjesbu (2001) and Friedland et al. (2005), we developed a lab set up that used an inexpensive flatbed scanner to capture images of oocytes and a freeware program to analyze the images. We then estimated fecundity using both the gravimetric and auto-diametric methods to determine the effectiveness of the procedure. The success of this procedure enables researchers to estimate fecundity of Atlantic cod using a simple sampling protocol and rapid laboratory procedure while incurring minimal equipment costs.

2. Materials and methods

2.1. Collection of samples

Ovaries of ripening Gulf of Maine cod were collected in January and May ($n = 46$ and $n = 45$, respectively) 2005 by bottom trawling. Because ovaries could not be weighed accurately at sea they were individually sealed in plastic bags, packed in ice and weighed in the lab 24 h later, to the nearest 0.001 g.

For each individual, two or three 0.1–0.2 g sub-samples of ovarian material were removed from the center of the right ovarian lobe and weighed to the nearest 0.001 g before being preserved. To take such small samples, first a larger amount of ovarian material was removed using a plastic syringe with the end cut off at the zero mark, then 0.1–0.2 g of material was scooped out of the end of the syringe using a stainless steel chemistry spatula, and weighed as it was added to a vial of preservative. Preservation entailed placing a sub-sample in a 10 mL clear plastic vial containing 5 mL of 10% phosphate-buffered formalin, capping and shaking the vial to ensure that all oocytes were thoroughly immersed, and storing the vial upright in a rack at room temperature. Samples were preserved for 4–5 months before being analyzed and were shaken periodically to aid preservation and oocyte separation. In previous research, this method of preservation was determined to be very effective when preparing cod oocytes for digital image analysis (Klibansky and Juanes, in review).

The methodology we developed for counting and measuring oocytes was largely based on the work of Thorsen and Kjesbu (2001). First a vial was shaken vigorously for 30–60 s to break apart any oocytes that were still connected. After shaking the vial, a 10 mL clear glass pipette and a plastic, thumb-wheel, pipette pump were used to carefully transfer all oocytes to a Petri dish containing ≈2 mm of 10% phosphate-buffered formalin. The Petri dish also contained a drop of soap solution (1 part Palmolive® antibacterial liquid dish soap to 20 parts distilled water) to reduce the surface tension of the liquid, making the oocytes spread out in the dish and keeping them from floating. Once a sample was plated out, the oocytes were swirled around in the dish to maximize dispersion, a black lid was then placed on the dish to serve as a contrasting background, and an image of the sample was captured using an Epson Perfection 1670 scanner flatbed scanner.

The largest image that the scanner could capture was 21.6 cm × 27.9 cm (8.5 × 11 in.) though at the desirable resolution, such an image would have been too large for the

computer's processor (Mobile Intel® Pentium® 4 CPU 3.06 GHz 1.59 GHz, 1.00 GB of RAM) to handle. Due to these processor limitations, samples were plated out into Petri dishes as described above, captured in Images 8.99 cm × 8.99 cm at 1600 dpi (5669 pixels × 5669 pixels) in 8-bit grey scale with a contrast setting of 18, and saved in uncompressed tagged image file format (TIFF). Once scanned, each 31 MB image was analyzed using the freeware program ImageJ Version 1.34j. This program is available for download at http://rsb.info.nih.gov/ij/. Additional downloadable plugins and instructional resources for ImageJ are available at http://www.uhnresearch.ca/facilities/wcif/imagej/index.htm.

2.2. Counting oocytes using ImageJ

Images scanned at 1600 dpi were converted to 600 dpi, allowing adequate detail for counting while reducing the file size to 4.3 MB to reduce the load on the computer's processor. Once the image size was changed, the Petri dish was removed from the image leaving only pale oocytes against a dark background. Usually a few oocytes were stuck to the Petri dish and missed by the selection area, but were later accounted for.

Before ImageJ would accurately count oocytes, each image was modified in several ways. First, we used the 'Threshold' process to separate oocytes and debris from the background. The upper end of the threshold range was always set to the maximum, 255, while the setting for the lower end of the range was determined with some scrutiny, and generally set between 50 and 65. Once the range was chosen, the process was run changing pixels within the range to black and changing all other pixels to white leaving an image of black oocytes scattered on a white background. Tiny bits of debris remaining in the foreground were then removed using the 'Despeckle' process. Even in this cleaner image, many oocytes were still clumped together and would not be identified by ImageJ. Clumps were broken apart with a process called 'Watershed Separation', which identified most individual oocytes within clumps and separated them with a line one pixel wide. At this point, the image was ready for counting.

The size range of oocytes to be counted by ImageJ was set to 300–900 μm in diameter, which was narrower than the actual range of possible oocyte sizes, but produced a more accurate count than a wider range, and oocytes that fell outside the range were manually counted later. The 'Analyze Particles' process was then run to count oocytes, and produce a new binary version of the original image showing black ellipses against a white background, at each locus in the image where a particle was counted. By then reopening the original unmodified image in a new window and resizing it so that both images were the same size, a composite image was made showing the ellipses encircling particles that had been counted. The composite was created so we could see which oocytes ImageJ had counted and which it had missed. Counts were corrected by carefully scrutinizing each composite image and marking false positives and false negatives using the 'Cell Counter' plugin (available for download at http://www.uhnresearch.ca/facilities/wcif/imagej/index.htm). The number of false positives was then subtracted from the number of false negatives and this difference added to the ImageJ count resulting in the adjusted count.

2.3. Estimating mean oocyte diameter using ImageJ and Excel

When an image was opened, ImageJ automatically identified the scale as 1600 pixels/in. (0.06299 pixels/micron). We then changed settings so that area (total pixel count) and circularity (4π(area/perimeter^2)) were measured for each particle identified by ImageJ. Since measurements would be performed on high-resolution (1600 dpi) images, we selected only a portion of each image, one third to one half of the contents of the Petri dish, and cropped the image. The remaining cropped image contained hundreds or thousands of pale oocytes on a dark background representing a random sample of oocytes from the ovary.

The 'Threshold' process was then used, as in the counting procedure, to differentiate oocytes and debris from the background. We manually adjusted the threshold range for each image and set the size range of particles to be measured to 200–1800 μm in diameter. This range was actually set wider than the expected size range of vitellogenic oocytes, so that if very large or small oocytes were present in the sample, they would be measured and identifiable. The 'Analyze Particles' process was then run to measure all particles and display the measurements in the 'Results' window. These results were then copied and pasted into an Excel file.

The entire measurement process was then run again for a second sample of oocytes from the same portion of the same ovary. Those measurements were also copied and pasted into the same Excel file.

The two sets of data pasted into Excel consisted of three columns each: (1) measurement ID number, (2) area, and (3) circularity. A measurement ID number was assigned to each measured particle in the order it was measured. ImageJ measured area by counting the number of adjacent pixels that compose each particle. Circularity was calculated by ImageJ as 4π(area/perimeter^2) while perimeter was the length of the outside boundary of a particle (Collins, 2006). A macro, written using Excel's Macro Recorder feature, was then run on both sets of data to filter out particles with a circularity value <0.8. Particles that were filtered out were usually small clumps of oocytes, broken oocytes or debris. Then using the area measurement and the formula for the area of a circle, the diameter of each oocyte was calculated as $2((\text{area}/\pi)^{1/2})$. The macro then used the first 100 diameter measurements from each filtered data set (i.e. 200 total) to generate a suite of descriptive statistics, including mean and standard deviation (S.D.). It also calculated these statistics for the first and second sample separately, and calculated the coefficient of variation (CV) between the means of the samples, as $100\text{S.D.}(\text{Mean}_{\text{Sample1}},\text{Mean}_{\text{Sample2}})/\text{Mean}(\text{Mean}_{\text{Sample1}},\text{Mean}_{\text{Sample2}})$ used to assess how similar the data sets were. Finally, the macro generated a percent-frequency histogram for the entire set of 200 measurements (Fig. 1). This histogram was used to assess the data for bimodality and outliers, and also to check that the size range of oocytes measured by ImageJ captured the entire cohort of vitellogenic oocytes.

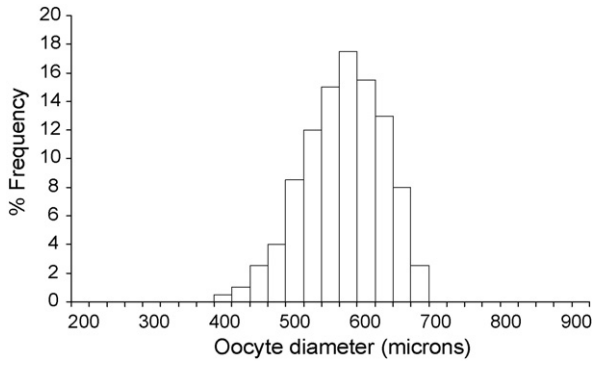

Fig. 1. Sample histogram of oocyte diameter and percent frequency of occurrence for an individual fish, $n = 200$ oocyte measurements.

2.4. Estimating potential fecundity using the gravimetric method

All vitellogenic oocytes in a 0.1–0.2 g sample of ovarian material were counted using ImageJ, then oocyte density was calculated by simply dividing the count by the weight of the sub-sample. Oocyte density was calculated for two sub-samples for each ovary, and the CV for these samples was calculated. Following Kjesbu et al. (1998), a specimen was only included in fecundity analyses if the CV of the sub-samples was 5% or less, to assure a high level of precision of the fecundity estimates. Potential fecundity ($F_{\text{Potential}}$) was then calculated by multiplying oocyte density (O_{Density}) by ovary weight (W_{Ovary}) but because whole ovary weight included the weight of the ovarian mesentery ($W_{\text{Mesentery}}$) it was adjusted by subtracting the estimated mesentery weight from the ovary weight. Mesentery weight was estimated using the relationship developed by Buzeta and Waiwood (1982), reprinted here as Eq. (2):

$$W_{\text{Mesentery}} = 0.03 W_{\text{Ovary}} - 2.37 \quad (2)$$

Potential fecundity ($F_{\text{Potential}}$) was then calculated using Eq. (3):

$$F_{\text{Potential}} = O_{\text{Density}}(W_{\text{Ovary}} - W_{\text{Mesentery}}) \quad (3)$$

2.5. Estimating potential fecundity using the auto-diametric method

Because the equipment used to capture digital images in this study was different than in the work of Thorsen and Kjesbu (2001), it was necessary to develop a function describing the relationship between mean oocyte diameter and oocyte density, using our particular equipment. Of 77 fish collected in 2005 for which both O_{Diameter} and O_{Density} were directly measured by the methods described above, 50 were selected randomly and used to develop this relationship. Following Thorsen and Kjesbu (2001) a power function of the form $y = ax^b$ was fit to the data using Microsoft Excel (Microsoft® Office Excel 2003, Microsoft Corporation), where y is O_{Density}, x is O_{Diameter}, and a and b are constants. We also tested whether skewness and kurtosis of the oocyte size distributions were related to mean oocyte size for these 50 observations using SAS version 9.1 (SAS Institute Inc., Cary, NC, USA). To do this, we first plotted each of these two variables against mean oocyte size to look for a relationship, then fit a line of the form $y = ax + b$ and conducted a test of the slope where $H_0{:}a = 0$. The remaining 27 observations were used to generate a scatterplot of fecundity estimates generated using the auto-diametric method versus estimates generated using the gravimetric method. We then fit a line of the form $y = ax + b$ to the scatterplot and conducted a test of the slope where $H_0{:}a = 1$ and a test of the intercept where $H_0{:}b = 0$ using SAS version 9.1.

3. Results

3.1. Relationship between oocyte size and oocyte density

We found the relationship between oocyte diameter and oocyte density (Eq. (4), Fig. 2) to be very strong.

$$O_{\text{Density}} = 1.182 \times 10^{12} O_{\text{Diameter}}^{-2.930}(\mu\text{m}) \quad (4)$$

$n = 50$, $r^2 = 0.971$, $350 \leq O_{\text{Diameter}} \leq 786\ \mu\text{m}$.

The relationship was similar and nearly as strong as that developed by Thorsen and Kjesbu (2001) (Eq. (1) of this manuscript). Substituting the right side of Eq. (4) for the value of O_{Density} in Eq. (3) produced the formula used to estimate potential fecundity by the auto-diametric method (Eq. (5)).

$$F_{\text{Potential}} = (1.182 \times 10^{12} O_{\text{Diameter}}^{-2.930}(\mu\text{m}))(W_{\text{Ovary}}(\text{g}) - W_{\text{Mesentery}}(\text{g})) \quad (5)$$

Analysis of the shape of the oocyte size distribution showed that mean oocyte size was not significantly related to kurtosis ($n = 50$, $r^2 = 0.068$, $P = 0.067$) (Fig. 3) or skewness ($n = 50$, $r^2 < 0.0001$, $P = 0.952$) (Fig. 4). Potential fecundity estimated by the gravimetric method ($F_{\text{Gravimetric}}$) was in good agreement with potential fecundity estimated by the auto-diametric method ($F_{\text{Auto-diametric}}$) (Eq. (6), Fig. 5).

$$F_{\text{Auto-diametric}} = 1.119 F_{\text{Gravimetric}} - 186062 \quad (6)$$

$n = 26$, $r^2 = 0.961$

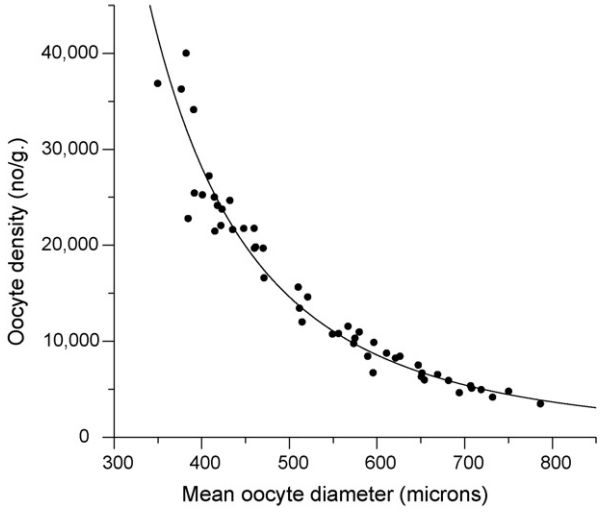

Fig. 2. Bivariate scatter of oocyte density and mean oocyte diameter. Oocyte density unit: number of oocytes per gram of ovarian material (no./g), $n = 50$ fish.

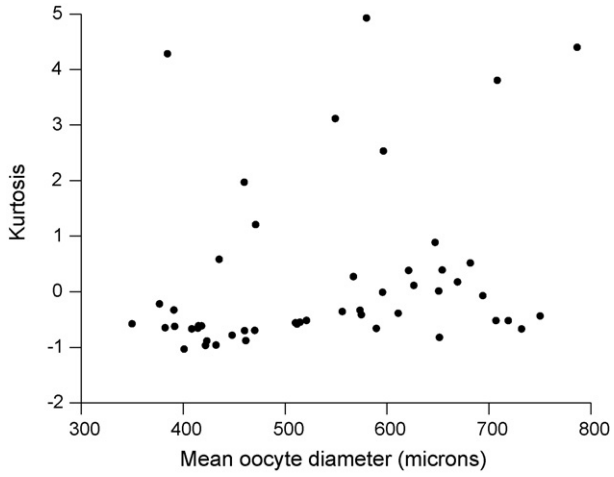

Fig. 3. Bivariate scatter of mean oocyte diameter and kurtosis of the distribution of oocyte diameters. Measurements based on 200 oocytes per fish, $n = 50$ fish.

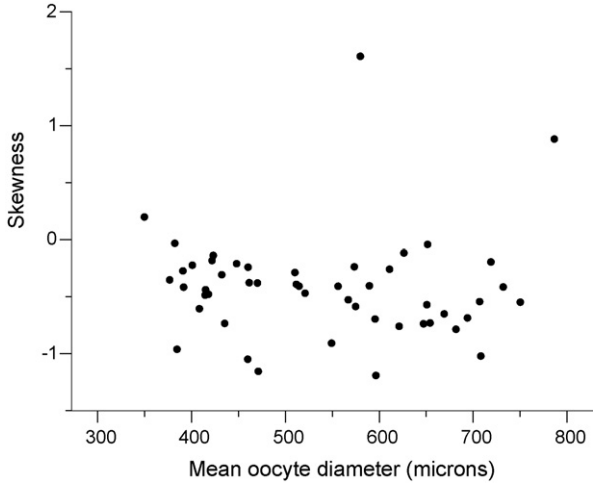

Fig. 4. Bivariate scatter of mean oocyte diameter and skewness of the distribution of oocyte diameters. Measurements based on 200 oocytes per fish, $n = 50$ fish.

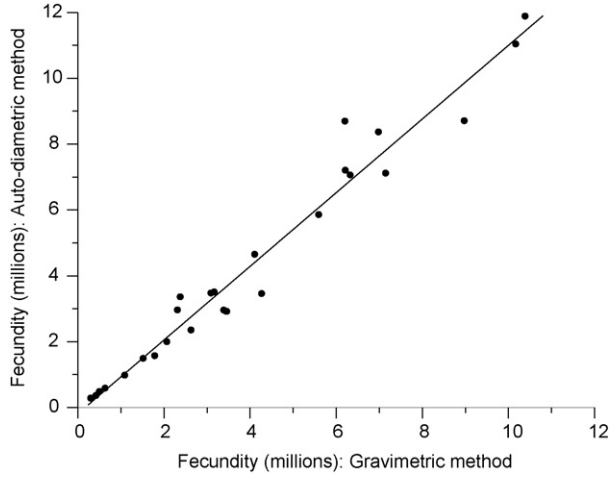

Fig. 5. Bivariate scatter of potential fecundity estimated by the gravimetric method and the auto-diametric method, $n = 26$ fish.

The 26 observations included in Eq. (6) were not included in developing Eq. (4). One additional observation not used to develop Eq. (4) was an outlier that exhibited high influence on the regression and was excluded. When the outlier was included the r^2 value of the fitted line was only slightly different (0.965). With the outlier excluded, the y-intercept of Eq. (6) was not significant ($P = 0.404$), and the slope was not significantly different from 1 ($P = 0.263$), suggesting that Eq. (6) is essentially the line $y = x$ and that $F_{\text{Auto-diametric}} \approx F_{\text{Gravimetric}}$.

Counting of oocytes took approximately 15 min for each 0.1 g sample for specimens with smaller oocytes and as little as 15 min for each 0.1 g sample for specimens with larger oocytes. The time taken to set up and execute the automatic counting portion of the procedure was only 1–2 min, regardless of oocyte size, but automatic counting was less accurate at counting small oocytes and manual correction of the counts for these specimens took considerably longer. However, exclusion from analyses due to imprecision (CV > 5) between sub-sample counts was not related to mean oocyte size, suggesting that manual correction of automatic counts was effective and unbiased.

Because accurate estimation of oocyte density required all oocytes in a pre-weighed sub-sample to be counted, handling of these sub-samples required more care and time (≈10 min) than for those that simply needed to be measured (≈5 min). But once the relationship between mean oocyte size and oocyte density was established, use of the auto-diametric method was appropriate and fecundity was estimated more rapidly for the remaining samples. Using the auto-diametric method, the entire process of removing a sample from a vial to producing data took approximately 10 min/sample by the end of the study. If many more samples were analyzed, processing time would likely have decreased as lab technician experience increased. But given the relatively low number of samples we needed to process in a day, 10 min/sample was sufficiently rapid, and allowed time for careful processing and data management.

4. Discussion

The effectiveness of the auto-diametric method is dependent upon a precise estimate of the relationship between mean oocyte size and oocyte density. We were able to fit a power function to our data to describe this relationship with a very high level of precision (see Eq. (4), Fig. 2), similar to that of the relationship developed by Thorsen and Kjesbu (2001) (Eq. (1) of this paper). Furthermore we found that $F_{\text{Gravimetric}}$ was in good agreement with $F_{\text{Auto-diametric}}$ and for the sample of $n = 26$ observations $F_{\text{Auto-diametric}} \approx F_{\text{Gravimetric}}$, suggesting that the relationship we developed can be used to estimate fecundity as precisely as the gravimetric method. The success of this relationship contrasts with that of Friedland et al. (2005). This may be due to differences in species studied and preservatives used.

While much of our methodology was based on Thorsen and Kjesbu's work, we used a flatbed scanner to capture images following Friedland et al. (2005). The standard practice in image processing is to capture images using a digital video camera and stereomicroscope (Russ, 1999) as Thorsen and Kjesbu did. But using a flatbed scanner has several advantages over the stan-

dard, including the simplicity of the image capturing procedure since light levels can be easily held constant within the scanner. The large field of view allows more oocytes to be captured in one image than when using a typical stereomicroscope. Flatbed scanners also cost much less than a digital video camera and stereomicroscope.

The scanner was very effective at capturing images of oocytes at the level of quality we required, though we highlight one disadvantage. The effective range of resolution of the scanner was not as broad as the advertised range when scanning oocytes in a Petri dish, such that image detail did not improve when image resolution was set higher than 3200 dpi. Because of the inherent limitations of the computer's processor, image resolution was set considerably lower than that when capturing images to be analyzed with ImageJ. Still, higher image detail would have been useful when investigating features of individual oocytes and would have been necessary if we had needed to count or measure oocytes smaller than about 150 μm in diameter.

The success and precision of this methodology is especially valuable to the field of fecundity research because of the low cost of the laboratory set up. Had we chosen a different combination of digital imaging software and laboratory equipment based on what other fecundity researchers are now using, we could have easily spent $10,000 instead of $100. We acknowledge that there may be certain gains to using more expensive software and equipment, and some laboratories no doubt require it for other purposes. Still, we emphasize that using our setup one can generate precise fecundity estimates using a simple, rapid, procedure, at a small fraction of the cost.

While once time and labor intensive, new methodologies make the task of gathering fecundity data much more reasonable to accomplish. Building on recent advances, our contribution has the potential to accelerate fecundity research by enabling scientists to efficiently produce high quality fecundity data on a very small budget.

Acknowledgements

We would like to thank Russell Brown of the Northeast Fisheries Science Center, Cpt. Jim Ford, Cpt. Russell Sherman, Bill Hoffman of the Massachusetts Division of Marine Fisheries, Cpt. Mike Russo and Cpt. Ted Ligenza of the Cape Cod Commercial Hook Fishermen's Association for providing us with access to ovary samples. We thank Andrei Klibansky and Lara Jarvis for laboratory assistance. We would also like to thank Kevin Friedland and Eva Garcia-Vazquez for their suggestions and comments. Funding for this study was provided by the National Oceanic and Atmospheric Administration, National Marine Fisheries Service, through the Cooperative Marine Education and Research Program (CMER) at the University of Massachusetts, Amherst. F.J. was also supported by a fellowship from the National Center for Ecological Analysis and Synthesis, a Center funded by NSF (Grant #DEB-0553768), the University of California, Santa Barbara, and the State of California.

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