

Abstract—Estimation of individual egg production (realized fecundity) is a key step either to understand the stock and recruit relationship or to carry out fisheries-independent assessment of spawning stock biomass using egg production methods. Many fish are highly fecund and their ovaries may weigh over a kilogram; therefore the work time can be consuming and require large quantities of toxic fixative. Recently it has been shown for Atlantic cod (*Gadus morhua*) that image analysis can automate fecundity determination using a power equation that links follicles per gram ovary to the mean vitellogenic follicular diameter (the autodiametric method).

In this article we demonstrate the precision of the autodiametric method applied to a range of species with different spawning strategies during maturation and spawning. A new method using a solid displacement pipette to remove quantitative fecundity samples (25, 50, 100, and 200 milligram [mg]) is evaluated, as are the underlying assumptions to effectively fix and subsample the ovary. Finally, we demonstrate the interpretation of dispersed formaldehyde-fixed ovarian samples (whole mounts) to assess the presence of atretic and postovulatory follicles to replace labor intensive histology. These results can be used to estimate down regulation (production of atretic follicles) of fecundity during maturation.

Manuscript submitted 2 May 2008.
Manuscript accepted 3 October 2008.
Fish. Bull. 107:148–164 (2009).

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Advances in methods for determining fecundity: application of the new methods to some marine fishes

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Research on population fecundity (total egg production) has two important applications in the management of renewable marine or freshwater fish resources. Perhaps the most important is to understand the relationship between spawning stock biomass and recruitment because it is increasingly clear that the assumption of direct proportionality (Beverton and Holt, 1957) is not correct (Marshall et al., 1998; Witthames and Marshall, 2008). The link between spawning stock biomass and recruitment varies according to total egg production that in turn is dependent, not only on the length frequency of spawning adults, but also on body weight at length (Marshall et al., 1998, 1999). In summary the stock and recruitment relationship became stronger when stock was expressed as a product of population length frequency and fecundity at length (Marshall et al., 1998).

A second application for fecundity information is to estimate spawning stock biomass independently of data collected from commercial fisheries (Parker, 1980; Lockwood et al., 1981; Lo et al., 1992). In addition to the fecundity count, information is

required on a range of parameters associated with the development of fecundity, such as follicular diameter and frequency distribution, spawning rates, and individual realized fecundity, either in one or multiple batches shed during one or more spawning events.

In this article we prefer the following definitions for fecundity (Hunter et al., 1992). Thus the developing fecundity (standing stock of fecundity referred to as “fecundity” [F]) includes follicles containing cortical alveoli (Khoo, 1979) and, in later development, yolk granules (Hunter et al., 1992) but excludes precursor cells such as previtellogenic follicles (PVFs) or oogonia. Relative fecundity (F_{bw}) is the fecundity divided by the total fish weight. We use the term “follicle” to refer to the oocyte and its nurturing follicular layers (Tyler and Sumpter, 1996) during all phases of development from precursor cells to residual postovulatory follicles (POFs) that indicate previous spawning or egg release events.

The species in this study are of interest because they represent three extremes in spawning strategy (Mu-

rua and Saborido-Rey, 2003): 1) group synchronous determinate total spawners (Atlantic herring [*Clupea harengus*], deep water redfish [*Sebastes mentella*] also known as "beaked redfish [FAO, Fisheries and Aquaculture Dept., www.fao.org/fishery/statistics/programme/3.1.1. Accessed Jan., 2009], and golden redfish [*Sebastes marinus*]); 2) group synchronous determinate batch spawners (Atlantic cod [*Gadus morhua*] and European plaice [*Pleuronectes platessa*]); and 3) asynchronous types (European hake [*Merluccius merluccius*] and Atlantic mackerel [*Scomber scombrus*]) that may not be determinate (Greer-Walker et al., 1994). Fecundity in the first two groups included all follicles in the advanced mode to the right of a gap in the follicular size frequency (Hunter et al., 1992) whereas in the latter case the follicular distribution is continuous. Although fecundity may be enhanced during maturation in asynchronous spawning types (indeterminate spawning strategy), it is of practical and theoretical value to study fecundity proliferation whatever classification is applied to the spawning process.

Recent work has shown that not all the fecundity develops into eggs (realized fecundity) and follicular atresia may account for a substantial part of the fecundity in a process referred to as down regulation (see reviews Murua et al., 2003; Thorsen et al., 2006; Kjesbu and Witthames, 2007). In addition, it is also important to differentiate whether an individual female has entered the spawning cycle, thus reducing her fecundity, and how long a POF persists to indicate a previous spawning event (Hunter and Macewicz, 1985a). The latter information is used to assess whether a female still contains her full complement of oocytes for application of the annual egg production method to assess spawning stock biomass applied to fish with a determinate spawning strategy (Armstrong et al., 2001).

To date no single approach has been successful in quantifying follicular stages associated with fecundity development and regression and each has one or more disadvantages. Fish that are very fecund, perhaps containing ovaries weighing more than a kilogram and with millions of follicles, will have to be subsampled for fecundity estimation. In this case, quantitative histological methods (Emerson et al., 1990) requiring sections of the whole ovary are not feasible—meaning only relative proportions of each follicular class can be measured (Andersen, 2003). This approach, however, needs additional information on the fecundity count preferably coupled with measurement of follicular size frequency to exclude smaller PVFs that are not committed to maturation in the current reproductive year. Although it is feasible to release follicles by digesting the ovary in strong acid solutions (either Gilson's fluid (Simpson, 1951) or a less toxic nitric acid formulation (Friedland et al., 2005)), such media have several adverse consequences. These consequences include 1) considerable follicular shrinkage (Witthames and Greer-Walker, 1987), 2) likely loss of atretic follicles and POFs (Klibansky and Juanes, 2007), and 3) incompatibility with histological methods (Hunter and Macewicz, 2003). In view of the need to

identify fecundity based on follicular size, there is a need to measure large numbers of follicles greater than a specified lower size limit even if the ovary is subsampled using the gravimetric method (Bagenal and Braum, 1968). Manual measurement of follicular size frequency, even using video technology, is just too demanding on manual labor unless there is some way of automating the collection of data. Although an automatic particle analyzer can provide such data (Witthames and Greer-Walker, 1987), the method requires large quantities of Gilson's fluid and is subject to all the problems listed above. More recently image analysis methods have been adopted to automate collection of size frequency data in Atlantic cod (*Gadus morhua*) (Thorsen and Kjesbu, 2001; Klibansky and Juanes, 2008). In each case the mean fecundity (the independent variable) can be used to estimate the number of follicles per gram (g) of ovary by fitting a power relationship based on a calibration from a data set containing the two variables (the autodiometric method). Fecundity is then determined by raising the number of follicles per g of ovary by the ovarian weight. Although an alternative image analysis method applied to American shad (*Alosa sapidissima*) (Friedland et al., 2005) has advantages as a cost effective method to estimate fecundity, it also has two significant drawbacks: 1) relatively low resolution, and, 2) it uses acid hydrolysis to separate follicles. Thus, the autodiometric method has more general utility because it uses neutral buffered formaldehyde solution (NBF) to fix tissue that is fully compatible with histology. Also Hunter et al. (1992) studying Dover sole (*Microstomus pacificus*) and Öskarsson et al. (2002) studying Atlantic herring (*Clupea harengus*) have shown it possible to identify atretic follicles in NBF-fixed dispersed ovarian samples (whole mounts) suggesting it might be possible to also estimate numbers of different follicular classes.

Accordingly, our first objective is to report on the utility and precision of the autodiometric method to determine fecundity in several species including Atlantic cod, European hake, Atlantic herring, Atlantic mackerel, redfish species (deep water redfish and golden redfish), and European plaice. To emphasize the utility of the method several laboratories 1) AZTI [A] (Pasaia, Spain), 2) Cefas [B] (Lowestoft, UK), 3) CSIC [C] (Vigo, Spain), and 4) IMR [D] (Bergen, Norway) used different configurations of image analysis equipment. In order to complete this work, four other objectives were identified linked to the application of fecundity determination using Atlantic cod as the main example and to a lesser extent European hake: 1) ovarian sampling, and follicular homogeneity, 2) evaluate three stains (eosin, rose bengal, and periodic acid-schiff [PAS]) to improve the accuracy of follicular size measurement and counting in relation to the autodiometric method, 3) compare interpretation of NBF-fixed whole mounts with respect to histology to assess maturity, spawning status and quantify the standing stock of atretic follicles, and 4) consideration was also given to the effect of ovarian maturation on down regulation of fecundity in Atlantic

Table 1

Details of the collection date, source, and maturity stage of wild fish (Atlantic cod [*Gadus morhua*], haddock [*Melanogrammus aeglefinus*], European hake [*Merluccius merluccius*], Atlantic herring [*Clupea harengus*], Atlantic mackerel [*Scomber scombrus*], European plaice [*Pleuronectes platessa*], common sole [*Solea solea*], redfish (deep water redfish [*Sebastes mentella*] or golden redfish [*Sebastes marinus*]) used in the study. The samples were taken from commercial or research vessel catches. Collections made in 1995 were used for the study of fecundity down regulation and the later collections from 1998 onward for the study of fecundity methods.

	Atlantic Cod	Haddock	European hake	Atlantic herring	Atlantic mackerel	European plaice	Common sole	Redfish
Date	Jan–Mar 1995, 2003–04, 2007	Feb 2007	Mar 2003	Jan 1998	Mar 2004	Jan–Mar 1995, 2000, 2007	Jan–Feb 1995	Sep–Nov 2000–2001
Source	Lofoten Andenes Norway North and Irish Sea	Irish Sea	Galicia Biscay Celtic Sea	Norwegian-spring spawning stock	Western Atlantic stock	Irish Sea	Irish Sea	Iceland Irminger Sea
Maturity stage	Prespawning and spawning	Pre-spawning	Pre-spawning and spawning	Pre-spawning	Pre-spawning	Pre-spawning	Pre-spawning to near spent	Pre-spawning

cod, European plaice, and common sole (*Solea solea*) and the implications for estimating total fecundity prior to the start of spawning.

Materials and methods

Ovarian sampling, follicle measurement equipment, and homogeneity

Ovarian samples were collected by the four institutes working on two or more of the following species Atlantic cod, Atlantic herring, European hake, Atlantic mackerel, European plaice, and redfish (including deep water redfish and golden redfish) for studies unrelated to this paper (Table 1). Biological information was taken from each fish, but only the information related to this method development (ovarian mass and maturity stage) is used here. Only active ovaries (Hunter et al., 1992) were selected and weighed to better than 2% of their mass, either with a motion-compensated balance (POLS Electronics, Isafjordur, Iceland) when sampled at sea or with a standard balance when on shore. Fish that contained many ovulated eggs (caught in the act of spawning) were not used in the autodiometric calibration because they show a heterogeneous distribution (Witthames, 2003). In each case ovaries or ovarian subsamples were fixed in a minimum of two volumes of NBF for a minimum of 14 days. Quantitative subsamples were taken by one of two methods: 1) from the fresh unfixed ovary (Atlantic cod, Atlantic mackerel) immediately after capture at sea using a Wiretrol II pipette (Drummond Scientific, Broomall, PA), or 2) from the fixed ovary in the laboratory with a scalpel (Atlantic cod, European hake, European plaice, and redfish). The Wiretrol II pipette

consists of a Teflon-tipped stainless steel piston within a graduated glass tube with a 1 or 2 millimetre (mm) bore that can remove 26 and 54, or 106 and 212 milligrams (mg), respectively, of tissue when inserted through the ovarian tunica.

Image analysis hardware and software, including the camera resolution and light intensities used by each institute to measure follicular diameter and circularity, varied (Table 2). The follicle data were analyzed with Microsoft Excel to calculate follicular mean, standard deviation, and leading cohort (*Lc*) (defined as the mean of largest 10% of follicles measured). PVFs were excluded from the fecundity count and frequency distribution based on a minimum follicular diameter of 150 and 250 μm in European hake, and Atlantic cod, respectively (Kjesbu, 1991; Murua and Motos, 2006). In the case of Atlantic mackerel there were no published data available, so a diameter of 185 μm was used based on our observation of the smallest follicles containing cortical alveoli and a publication focusing on Atlantic mackerel fecundity determinacy (Greer-Walker et al., 1994). In all other species, where the follicular frequency was not continuous, only dark yolk-bearing follicles in the leading mode were included in the fecundity count. Follicles were disaggregated from the ovarian sample by sucking them in and out of a Pasteur pipette (Thorsen and Kjesbu, 2001) prior to spreading them out in a counting chamber as a single layer completely covered by water. If small clumps of follicles remained they were measured manually as discrete follicles, whereas larger clumps were separated and exposed to more pressure washing by the pipette or a garden spray (Institutes A and C). All the follicles were counted in the subsamples but the method for collecting the follicular measurements differed according to each institute's image

Table 2

Details of the image analysis software (¹Pilkington Image Analysis Systems, ²Shareware available at rsb.info.nih.gov/ij/download.html), operating systems, camera, light source, microscope, resolution, grey scale, and staining methods used by each institute: AZTI, Cefas, CSIC, and IMR to analyze whole mounts prepared from each species: Atlantic cod (*Gadus morhua*), European hake (*Merluccius merluccius*), Atlantic herring (*Clupea harengus*), Atlantic mackerel (*Scomber scombrus*), European plaice (*Pleuronectes platessa*), redfish (deep water redfish (*Sebastes mentella*) or golden redfish (*Sebastes marinus*)).

Detail	AZTI [A]	Cefas [B]	CSIC [C]	IMR [D]
Software	Visilog 6.11	Myrmica ¹ automatic or semi automatic operation	QWin (Leica Imaging System)	Image SXM v. 1.77 ²
Operating system	PC Windows	PC Windows	PC Windows	Mac OS X
Camera	Camedia-4040 Zoom	Pulnix TMC-1000-CL	Leica IC A	JVC TK-1070E
Light source	3100 High light Olympus	3100 Olympus High light	Leica MZ6	SZX-ILLB200
Microscope	Olympus SZX12	Olympus SZX12	Leica MZ6	Olympus SZX12
Resolution ($\mu\text{m}/\text{pixel}$)	5 (hake)	19.3 (plaice), 10.0 (cod), 4.6 (mackerel)	21.9–27.6 (redfish) 7.02–22.43 (hake)	14.7 (cod) 14.7 (herring)
Gray scale (255 saturated)	230	175	90	205
Staining method	Rose bengal (hake)	PAS (mackerel and cod) Eosin (plaice)	Rose bengal (hake) Unstained redfish	Unstained

analysis system and the size of the follicles comprising the fecundity. Institutes A and C split the sample and took a separate image of each aliquot to ensure that the follicles were evenly spread without overlap in a container that was completely covered within the field of view. Institute B spread the sample in a counting chamber 70 mm long and either 4, 7, or 10 mm wide for Atlantic mackerel, Atlantic cod, and European plaice, respectively. The three widths of counting chamber were used so that three magnifications (Table 2) could be used while still displaying the full width of the chamber. Above the sample the counting chamber tapered outwards in v-shaped profile leading to an upper liquid surface of 25 mm creating a flat meniscus over the channel holding the follicles. Myrmica software (Pilkington image analysis systems, Lindfield, West Sussex, UK) was used to create and archive a series of images and overlays along the horizontal axis of the sample container showing all measurements overlaying the follicles measured. Individual follicles stained PAS were measured in this counting chamber both manually and by image analysis to establish the accuracy of size measurement by image analysis. Institute D working with Atlantic cod and Atlantic herring used a method described previously (Thorsen and Kjesbu, 2001).

In order to investigate whether fixing the ovary in sample aliquots or whole in the tunica affected mean follicular diameter (D_f , μm), circularity, and fecundity per gram of ovary (F_{ow}), replicate samples were taken by pipette and scalpel, respectively, from the central part of the same ovary from Atlantic cod, Atlantic haddock, and European plaice (Table 1). These samples were collected

from fish caught in the Irish Sea during February 2007 (Table 1) and fixed for between 63 and 91 days in 1.7 to 9 times their volume of NBF before image analysis (Table 2). Circularity, a function of follicular shape, was measured according to the following equation:

$$\text{Circularity} = 4\pi (\text{area}/\text{perimeter})^2. \quad (1)$$

Homogeneity of D_f and F_{ow} were studied in Atlantic cod and European hake ovaries to investigate whether a sample from the center of the ovarian mass was significantly different from samples taken at the extreme ends of each ovary or between the pair of ovaries.

Stain evaluation

Ovarian tissue (whole mounts) stained by the three routines (Table 2) was compared with unstained tissue in order to improve the identification and measurement of developing (cortical alveoli and vitellogenic) and regressing (postovulatory and atretic) follicles. Non-stained tissue was prepared for analysis as described by Thorsen and Kjesbu (2001), and two of the staining methods applied water soluble 1% eosin or 0.02% rose bengal weight to volume (w/v) dissolved in NBF to color the follicles. A third staining method used the PAS reaction, previously applied to stain cortical alveoli follicles (Greer-Walker et al., 1994). In this procedure the concentration of PAS reagent was 0.1% and 15% w/v, respectively, compared to the histological procedure in order to minimize shrinkage of follicles. Nonbound stain was removed from the tissue subsamples after

staining by washing through mesh sieves that retained all follicles larger than 125 μm using either 1:3 glycerine:water (McBride and Thurman, 2003), 0.9% w/v sodium chloride (Ramsay and Witthames, 1996), or clean water. Replacement of the fixative used for storage by saline or water did not affect the size of follicles during subsequent storage for 5 days at 0–5°C.

Comparison of whole-mount method with histological method

In order to study the morphology of POFs immediately after ovulation, and during advanced regression, ovarian samples were taken from trawl caught Atlantic cod taken from the Irish Sea (Table 1) during the spawning season. In some cases the fish ($n=10$) were producing copious quantities of ovulated eggs and were expected to contain newly produced POFs created simultaneously with ovulation. After fixation in NBF the whole mounts were examined both unstained and after PAS staining to color both the oocyte chorion and the basement membrane between the granulosa and thecal layers of the follicle. The size frequency of the residual vitellogenic follicles and POFs were also measured at this time. Normal vitellogenic and POFs were tentatively identified in the above preparations based mainly on their shape but also on their internal structure revealed as irregular blotches or shading (Hunter and Macewicz, 1985a, 1985b; Hunter et al., 1992). One fish was chosen from this group because it contained not only large POFs but also illustrated previous spawning activity based on large numbers of small POFs assumed to come from previous ovulation events. Examples of tentatively identified follicular classes were removed from the whole mount and processed into PAS Mallory trichrome stained 2-hydroxyethyl methacrylate (Technovit® 7100 Kulzer GmbH, Wehrheim, Germany) sections (Witthames and Greer-Walker, 1995) in order to compare the accuracy of the identification.

Alpha atretic follicles (Hunter and Macewicz, 1985b) were identified in biopsy samples taken with a Pipelle de Cornier® (Prodimed, Neuilly En Thelle, Picardie, France), a flexible, plastic tube 2.1 mm internal diameter, by endometrial suction after gonad catheterization (Bromley et al., 2000). These samples were taken from sedated captive Atlantic cod available from a separate study carried out at IMR during March 2004 to determine the rate of transition from normal to advanced stage atretic follicles. Each biopsy sample was fixed as above and examined as an unstained and stained whole mount to compare the intensity of follicular atresia found in both preparations. Intensity of atresia (Ia) was defined as

$$Ia = Ni/(Ni + Nj), \quad (2)$$

where Ni and Nj refer to alpha atretic and normal vitellogenic follicles, respectively.

The alpha atresia and more advanced beta follicular stages have been defined previously based on the frag-

mentation or absence of the chorion (Witthames and Greer-Walker, 1995; Witthames, 2003) following previous studies (Hunter and Macewicz, 1985b). After scoring the intensity of atresia the whole mounts were infiltrated in resin and then polymerized slowly at –10°C over a period of 2 hours that all the follicles lay at the base of the mold. At least 25 to 30 sections of 5 μm were cut and discarded in order to take a section within 125 to 150 μm from the base of the mold to transect all the follicles present in the sample. This section was stained by the PAS Mallory trichrome method to identify and count the transected follicles.

Fecundity maturation and down regulation

In order to study the change in fecundity during maturation D_f and atresia data were taken from Atlantic cod sampled in the Irish and North Seas between January and March during 2003 and 2004 (Table 1), and examined in two ways. In the first case the standing stock of atretic follicles (Ia) was measured as prevalence (proportion of fish containing alpha atretic follicles) and relative intensity (Ia /whole body weight g) as described previously (Witthames and Greer-Walker, 1995). The atresia was determined in histological sections stained by PAS Mallory trichrome. Secondly the overall impact of atresia on relative fecundity $F_{bw} = F/\text{total body weight in } g$ during maturation was determined by assessing the reduction of F_{bw} in relation to D_f as recently described (Thorsen et al., 2006).

$$F_{bw} = a \times \text{Ln}(D_f) + b. \quad (3)$$

An additional data set (Table 1) was also available from an annual egg production survey of Atlantic cod, European plaice, and common sole biomass (Armstrong et al., 2001) to assess whether down regulation also occurs in other species with a similar fecundity development process. This data set contained details of fecundity, fish length (cm) total, and ovarian weight (g) for each species and was used to calculate F_{bw} in each case. D_f was predicted from F_{ow} using the ovarian weight and fecundity data in Equation 4 (below) adjusted to make F_{ow} the independent variable.

Autodiametric calibration

A regression line (based on ln-transformed data) was established for each species and institute (Thorsen and Kjesbu, 2001) between D_f and F_{ow} using the following formula where a and b are equation constants.

$$\text{Ln } F_{ow} = a \times \text{Ln } D_f + b. \quad (4)$$

In one data set the parameters showed some degree of noncovariance and a second polynomial function ($\text{Ln } D_f^2$) was fitted to the data where a , b and c are constants:

$$\text{Ln } F_{ow} = a \times \text{Ln } D_f + b \times \text{Ln } D_f^2 + c. \quad (5)$$

Table 3

Comparison of mean (standard error [SE]) follicle diameter (D_f , μm), number of follicles per gram ovary (F_{ow}), circularity, and fecundity ($F_{ow} \times$ ovary mass [g]), found in samples taken with either a pipette (P, from fresh tissue) or gravimetric method (G, from fixed tissue) from fish caught in the Irish Sea by commercial vessel during 2007. In each case five replicates were taken from a central location of the same ovary in ripe mature Atlantic cod (*Gadus morhua*, cod m), hydrated Atlantic cod (cod h), ripe mature Atlantic haddock (*Melanogrammus aeglefinus*, had), and European plaice (*Pleuronectes platessa*, ple). The mass (g) of the ovary fresh (ovary F) and after storage in fixative for longer than 14 days (ovary S) is also shown.

Parameter	Sample method	Cod m (n=5)	Cod h (n=5)	Had (n=5)	Ple (n=5)	Paired <i>t</i> -test <i>n</i> =15 <i>P</i>
D_f	P	797 (3)	944 (5)	571 (4)	1081 (10)	0.0026
	G	777 (3)	904 (8)	560 (1)	1085 (7)	
F_{ow}	P	3613 (90)	1460 (338)	9355 (338)	1196 (38)	6.652 ⁻¹⁰
	G	3975 (149)	1706 (33)	10394 (149)	1280 (17)	
Circularity	P	0.986 (0.001)	0.995 (0.001)	0.985 (0.001)	0.990 (0.001)	0.0015
	G	0.966 (0.004)	0.983 (0.001)	0.961 (0.002)	0.977 (0.002)	
Fecundity	P	419,132	388,460	205,804	44,260	
	G	443,191	421,424	218,664	45,980	
Ovary	F	116.0	266.0	22.0	37.0	
Ovary	S	111.5	247.0	21.0	35.9	

Fecundity (F) was calculated from the product of ovarian weight (O) and F_{ow} .

Statistics

Regression analysis was carried out using the “R”(vers. 2.5.0, Free Software Foundation, Boston, MA) and residuals were plotted to check there was no systematic pattern suggesting that the models should be further refined. The coefficient of variation (CV) was determined for predictions with new data to examine the precision of the fecundity estimate for a range of follicular sizes typical for each species.

Results

Ovarian sampling, follicle measurement equipment, and homogeneity

In the course of more extensive use at sea, the pipettes performed well taking samples from maturing ovaries providing that they contained vitellogenic follicles visible to the unaided eye (>400 μm). However, ovaries that were close to being spent or immature did not yield quantitative samples because the connective tissue attached to the follicles pulled the sample out of the pipette as it was withdrawn from the ovary. When this occurred it was clear that the glass tube was only partially filled and the sampling process could be repeated to fill the pipette to avoid under sampling although this was not always successful. In summary we found that replicate subsamples of 25 and 100 μL tissue taken with the pipette from a Atlantic cod ovary equated to a gravimetric sample of 26.0 mg (CV=1.8%, *n*=10) and 106.0 mg (CV=3.7%, *n*=10), respectively.

Compared to fixing the ovary whole for the gravimetric method, the pipette procedure for collecting ovarian subsamples was found to significantly increase ($P=0.003$, $P<0.0001$, and $P=0.002$) D_f , circularity of follicles (Table 3), and decrease F_{ow} , respectively. Ovarian weight after fixation over 63–65 days showed a small decrease (95% SE=0.9) that was not apparently related ($P=0.55$, *n*=4) to the amount of NBF used to fix the ovary. After accounting for a reduction in ovarian mass the overall reduction in fecundity, determined from the pipette samples, was 5.7% (SE=0.3) less compared to gravimetric samples taken from the same ovary fixed whole.

There was a very significant difference in F_{ow} , D_f , and Lc means ($P<0.001$) between fish but there was no consistent trend either between the pair of ovaries or within the ovary at three sites (anterior, middle, and posterior) where samples were taken (Fig. 1). In two out of the seven fish there was a site effect (left, posterior, and right middle) on D_f and Lc , but their rank order was reversed at other locations. It was also noticed that in more mature Atlantic cod ovaries, where the Lc was larger, the CV of D_f and Lc amongst replicates also increased. Similarly, sampling site (anterior, middle, or posterior part of one ovary) in 103 European hake indicated that F_{ow} , either classified by cortical alveoli, early, or late vitellegenic follicle development stages, or all classes combined, was not related to ovarian position ($P=0.133$, 0.149, 0.789, 0.101, respectively).

Stain evaluation

Compared to unstained follicles the use of each stain to color European hake, Atlantic cod, Atlantic mackerel, and European plaice follicles increased the efficiency of image analysis measurement, particularly of semitrans-

parent objects such as PVF, cortical alveoli, hydrated, and POFs. After PAS staining, manual measurements of follicle diameter (F_{dm}) compared closely to automatic measured follicle diameter (F_{di}) calculated from the image analysis:

$$F_{di} = 1.0016 \times F_{dm} + 0.0405 \quad (6)$$

($n=42$, $r=0.997$, $220 \leq \text{manual reading} \leq 1900 \mu\text{m}$)

Although the eosin solution stained both vitellogenic and hydrated follicles in European plaice, it was much less effective when applied to either Atlantic cod or Atlantic mackerel follicles. A further disadvantage was that the stain was not bound by a chemical reaction and tended to leach out more rapidly compared to PAS stained tissue. This could be countered by extensive washing but this

progressively affected the follicle size determined by image analysis. The rose bengal stain was also based on affinity rather than chemically bound and excess stain had to be washed from the sample. It was an effective aid to automatic measurement of PVF and POFs from Atlantic cod, European hake, and Atlantic mackerel, though the coloration was not as intense compared to PAS. Also the PAS stain was particularly useful when applied to whole mounts from Atlantic cod making it easier to identify the outline of small POFs compared to PVF that were less intensely stained.

Comparison of whole-mount method with histological method

A whole mount prepared from a female Atlantic cod caught during ovulation (Fig. 2A) revealed small POFs from earlier ovulations (Fig. 2B left) and also much larger POFs (Fig. 2B right). The latter appeared as large round membrane structures, about the size of hydrated follicles formed by the thecal and granulosa layers that remain in the ovary to form the POF. A burst zone in the circular membrane was also visible, probably made during the expulsion of the ripe egg. POF shape and morphology was equivalent in whole mounts and section and characterised by deep red staining in section and denser grey scale in stained and unstained whole mount respectively (Fig. 2B left and middle). PVFs in unstained whole mounts appeared quite translucent with a central nucleus that was consistent with their shape and form in section and easily distinguished from POFs.

Atretic follicles were rather easily identified in unstained whole mounts and their morphology could be equated to that seen in histological section (Fig. 3). Comparing these preparations the chorion appeared to be progressively broken down and provided a useful criterion to identify late alpha atretic follicles in whole mounts. A comparison of alpha atretic intensity between the two methods (Fig. 4) indicated that the whole mount preparation could provide an indication of both prevalence and intensity of atresia.

Fecundity maturation and down regulation

Atlantic cod fecundity data from the North and Irish Sea collected in 2003 and 2004 (Table 1) was analysed to investigate whether the relative fecundity declined during maturation. As the ovary matured and D_f increased from 350 to 800 μm the prevalence of atresia increased (Fig. 5). Relative intensity of atresia was absent in ovaries with a D_f of 350 μm and tended to remain at a fairly low level but with one much higher value when the mean follicular diameter was 650 μm . Also during this maturation period there was a drop in predicted mean relative fecundity for all fish in the sample amounting to 49.6% whilst fecundity diameter measured manually or automatically (F_d) increased from 355 to 794 μm . Analysis of the data from the 1995 survey indicated that fecundity was overestimated between 11% (Atlantic cod) and 13% for European plaice

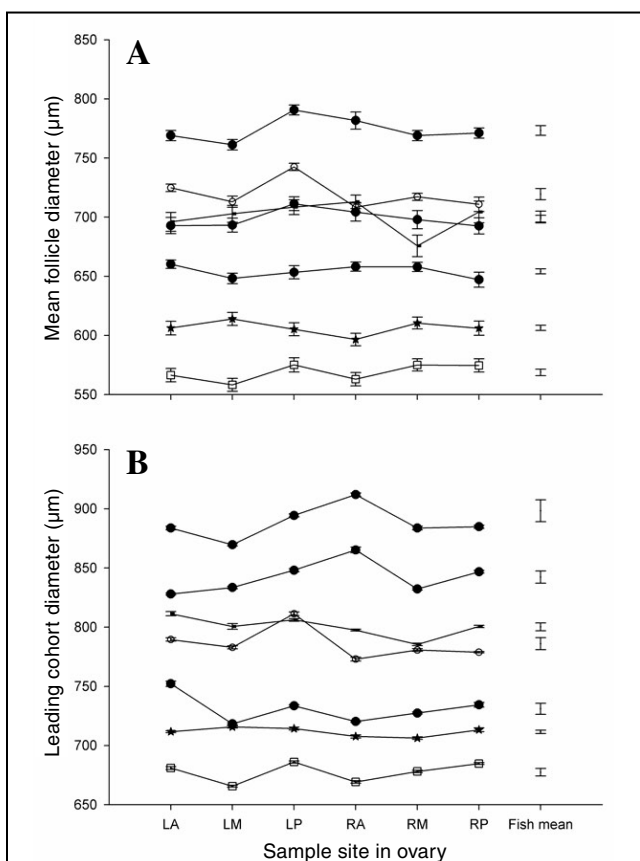
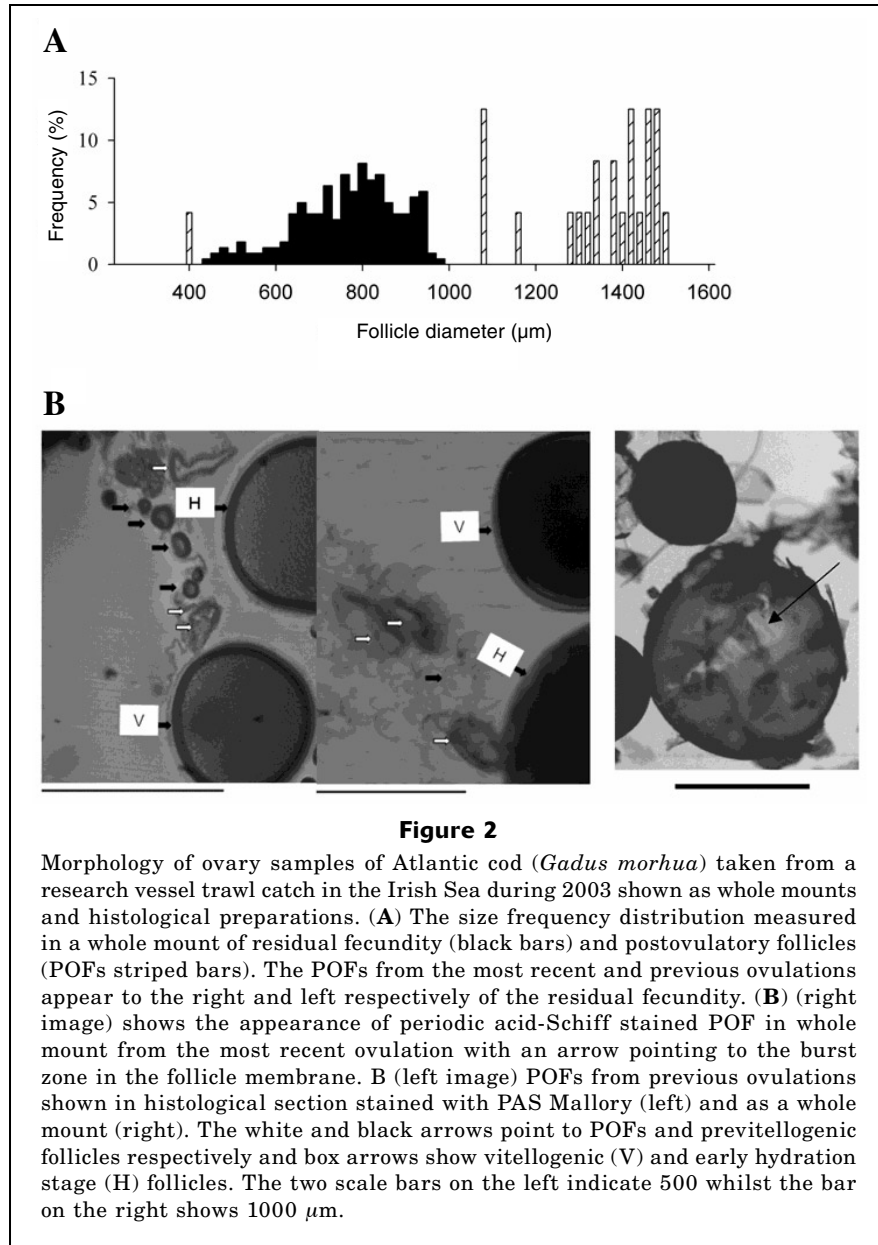


Figure 1

Comparison of mean follicle diameter (A) and leading cohort diameter (B) ± 2 standard error taken from three sites, anterior (LA and RA), middle (LM and RM), and posterior (LP and RP), in each pair (left L or right R, respectively) of seven ovaries of Atlantic cod (*Gadus morhua*) caught by commercial vessel using gill nets landing at Andenes, Norway in 2007 (Table 1). Lines join the sites for each fish. Approximately 200 unstained follicles were measured using image analysis by the Institute of Marine Research Norway at each site. Fish mean refers to the overall mean follicle diameter of all sites for each fish.



and common sole (Fig. 6, Table 4). From the perspective of fecundity methodology, the measurement of F_d as well as the standing stock of fecundity make it possible to adjust the fecundity to the same point in maturation, defined by mean follicle size, close to the start of spawning.

Autodiametric calibration

The seven species examined, even when in an advanced stage of maturity, contained very different forms of fecundity size frequency distribution (Fig. 7) ranging from normal (Atlantic herring, European plaice, and redfish) to a more skewed shape (European hake and Atlantic mackerel). In two species (Atlantic cod and

European plaice) samples with a hydrated, bimodal distribution were also included in the data set.

The equations (Table 5) from the regression analysis, based on the autodiametric calibration applied individually to Atlantic cod, Atlantic mackerel, Atlantic herring, European plaice, and redfish (Fig. 8) for each institute, made it possible to predict F_{ow} (Eqs. 4 and 5) with high precision in most cases (Table 6). European hake and Atlantic mackerel are examples where the vitellogenic follicle distribution is continuous extending down to overlap with the PVF population (Fig. 7) and produced a higher CV to predict F_{ow} from D_f using Equation 2. In the case of European plaice, Atlantic cod, and European hake, some ovaries contained both maturing and hydrated follicles

exhibiting a bimodal frequency distribution, but in each case the autodiometric calibration made it possible to make estimates of F_{ow} with an acceptable level of precision. Equation 5 gave a small but significant ($P < 0.0001$) improved fit, but only for Atlantic cod with hydrated follicles, and reduced the CV of F_{ow} estimates predicted from 450 to 1050 μm D_f . The overall precision after inserting an ascending series of D_f in Equations 4 and 5, spanning the range found in each species was always better than a CV of 3% based on a prediction for new data.

A combination of the data in a general calibration curve (Fig. 9A) is provided to show that the auto-diometric method has general application and may be used with other species. However when compared against the individual species model there was difference in the predictions by up to 20% both within species and between institutes (Table 6). The variance was greater in the fish with a continuous follicular distribution, especially in the case of European hake (Fig. 9, B and C).

Discussion

Our results show that the pipette method for sampling fresh ovaries at sea can be used to replace the need to return the whole ovary for the gravimetric fecundity method (Bagenal and Braum, 1968), provided ovarian weight can be recorded precisely onboard. Although the pipette fecundity was slightly lower (94.7%) compared to the gravimetric fecundity, we feel that the scale of difference can be easily nullified by a small correction factor and is small compared to the reported variability in fecundity over time (Rijnsdorp, 1991) and space (Witthames et al., 1995). Our confidence in making this statement is increased because of a direct comparison between both methods for the same ovary and because the autodiometric calibrations are very similar without large residuals attached to either method. A previous report described a cut down plastic syringe to suck up standard sized ovarian samples of 1.54 g (CV=3.7 $n=155$), but the commercially available alternative described in this paper has two advantages: 1) it is already calibrated for a range of sample sizes (25, 50, 100, and 200 μL), and 2) it is suited to taking small samples appropriate for fecundity determination in species such as Atlantic mackerel and European hake. In our results ovarian weight showed on average a small decline (-5%) from fresh to fixed weight for each species which was considerably different from a previous report (Klibansky and Juanes, 2007) at +5% or less. The reasons for the difference are not apparent but do not involve the ratio of fixative to weight of ovarian tissue because the range used in this work (1.7 to 9.1 times NBF to ovarian weight) spans the ratio of four times where a positive weight change was recorded.

Collection of fecundity samples in this way has clear advantages: 1) require small amounts (1.2 compared to more than 5000 ml for species like Atlantic cod) of NBF (classed as a carcinogen), 2) reduced exposure because of the smaller free surface for evaporation, 3) lower environmental impact for disposal of fixed tissue and waste fixative, 4) it is more feasible to collect fecundity samples on com-

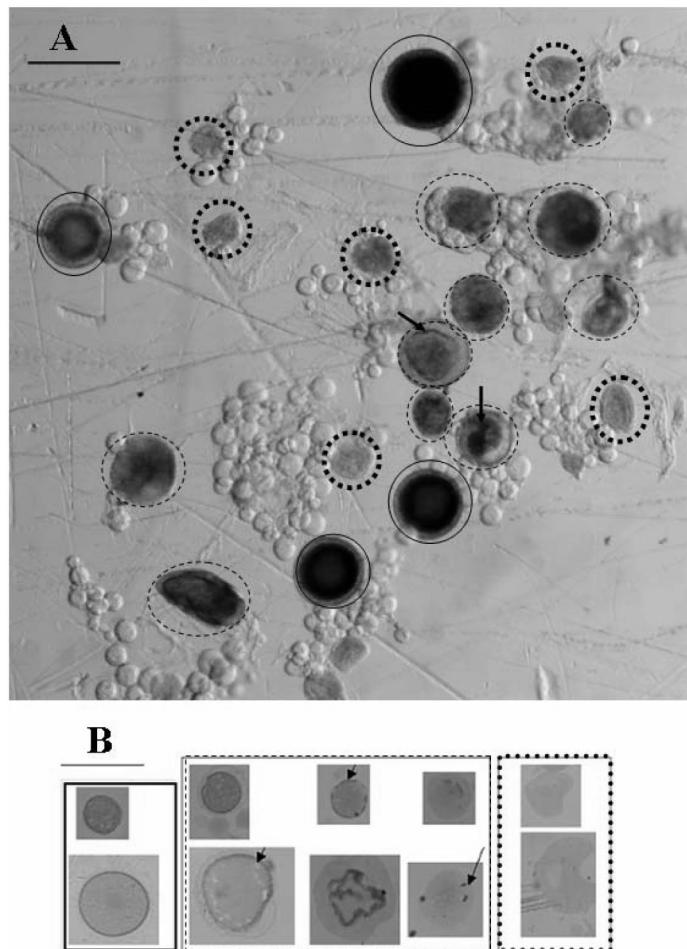
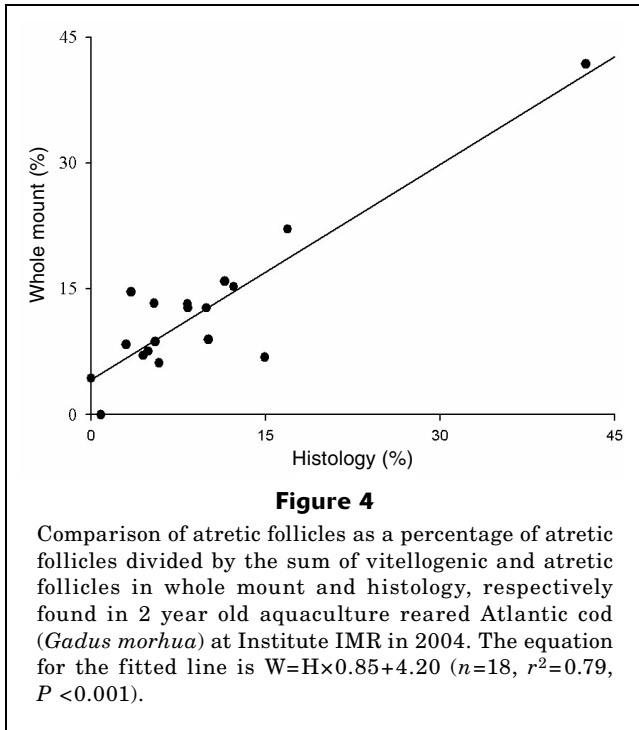


Figure 3

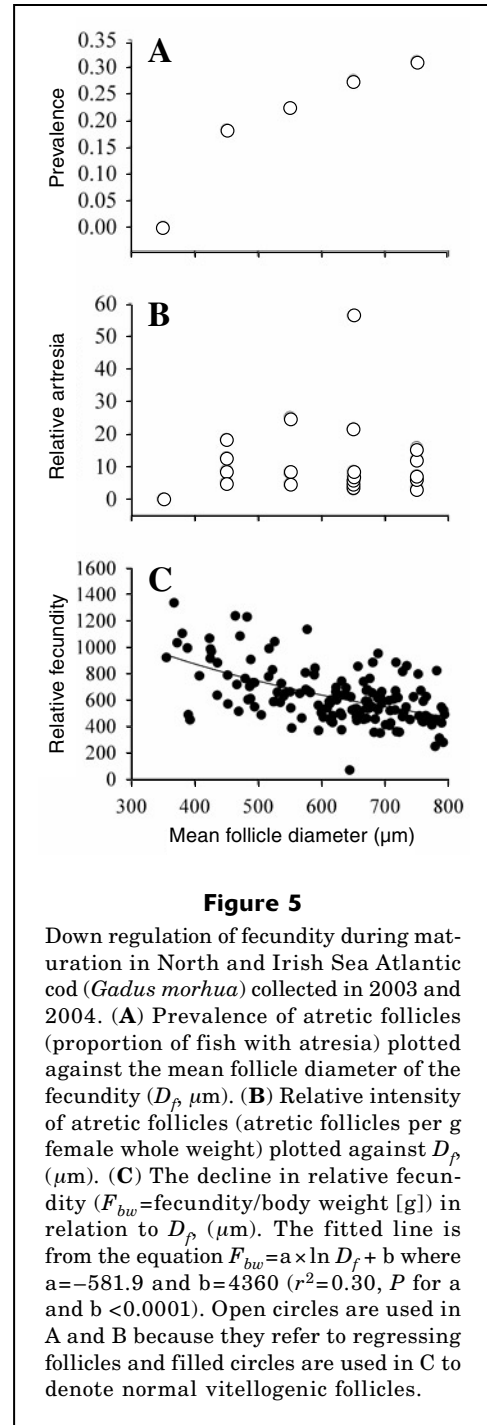
Appearances of atretic oocytes taken from 2 year old aquaculture reared Atlantic cod (*Gadus morhua*) at Institute IMR in 2004. (A) Image taken from an unstained whole mount prepared from an ovary biopsy containing high levels of alpha atretic follicles (dashed circles), beta atretic follicles (dotted circles), and normal vitellogenic follicles (black circles). (B) Histological section of the biopsy in A showing the same classes of follicle (outlined using the same line key as A) after processing into histological section. In each case arrows point to the disintegrating chorion used for classification of alpha atretic follicles. The scale bars (top left of A and B) = 500 μm .



mercial vessels, 4) easily portable samples that can be distributed at lower cost to facilitate exchange of samples between laboratories carrying out international egg production based stock assessments (Armstrong et al., 2001) and, not least, 5) better preservation of histological detail to identify and determine proportions of atretic and postovulatory follicles. Our finding that POF in recently ovulated ovaries are spherical and not collapsed, compared to previous studies, may be attributed to less compression in a biopsy compared to ovaries fixed whole.

A small difficulty in not returning the whole ovary back to the laboratory for subsampling means the ovary must be weighed at sea, sometimes in rough conditions, but this can be achieved using motion-compensated balances. Although this equipment is perhaps beyond some research budgets it has been successful even on commercial vessel in rough conditions providing the mass for weighing is less than 75% of the upper weighing range. Also balances are available with a resolution of 0.01 g making it feasible to weigh ovaries from probably all commercial species with an acceptable accuracy. If it is not feasible to use motion-compensated balances, it has been reported that ovaries can be returned for weighing after the end on of the cruise providing the ovary is packed to prevent water loss (Klibansky and Juanes, 2008). A further recommendation, in connection with using the pipette, is to complete at least duplicate samples and the follicles should not be larger than the pipette internal bore of 2 mm although exception can be made for hydrated follicles just larger than 2 mm.

In this study it was shown that the ovary is homogenous in regard to F_{ow} and D_f both for Atlantic cod and



European hake. Homogenous packing has also been reported for hydrated oocytes prior to ovulation in European hake (Murua et al., 2006). The posterior region of the ovary is the most variable and in some fish this part can be packed with significantly different sized follicles and should be avoided. However, it should not be assumed that F_d is universally independent of location because small differences (2%) in F_d heterogeneity have been reported in flatfish species such as yellowfin

sole *Limanda asper* (Nichol and Acuna, 2001) and European plaice (Kennedy et al., 2007). Samples used for the auto-diametric calibration and in subsequent determination of F_{ow} should have the same fixation history because fixing conditions affect D_f , F_{ow} , and circularity and also affects the ovarian weight (Klibansky and Juanes, 2007) used to raise F_{ow} to fecundity.

We would not recommend the general use of PAS stain for image analysis in mature fish because it obscures

the chorion detail which is used to classify atretic from normal vitellogenic follicles (Kjesbu et al., 1991). The main advantages of PAS, compared to the other stains evaluated, was that it was the most color fast, worked with all the species where it was tried, and provided specific staining to color more transparent objects such as cortical alveoli, hydrated, and postovulatory follicles. It is however more laborious to apply, but has been successful in all applications where it has been tried previously (Kennedy et al., 2007) and performed well in the comparison of manual versus automatic measurements. Similar results have also been found for nonstained follicles, although not reported in the results section.

Based on our results and earlier reports (Witthames and Greer-Walker, 1995; Kurita et al., 2003; Thorsen et al., 2006; Kennedy et al., 2007) fecundity is down regulated by the production of atretic follicles during maturation. If samples are taken close to spawning season, down regulation is not significant (Öskarsson and Taggart, 2006), but the timing of sampling should be considered especially when studying multiyear collections for example: Atlantic cod (McIntyre and Hutchings, 2003), European plaice (Horwood et al., 1986; Rijnsdorp, 1991) and common sole (Witthames et al., 1995). Using the autodiametric method, it was possible to predict D_f providing data on ovarian weight and fecundity is reported using a rearranged Equation 4. This method was used in this study for 1995 survey data to standardize fecundity for maturity and indicated that the spawning stock biomass of Atlantic cod, European plaice, and common sole may have been overestimated by about 12% based on follicle diameters of 650, 1100, and 600 μm , respectively. Although we consider that follicular atresia was an important cause of negative fecundity residuals in this study, we do not exclude an alternative explanation that more fecund individuals within a fecundity sample produce smaller eggs, and vice versa (i.e., a trade off between fecundity and egg size). Such a trade off is likely in a comparison between stocks such as Atlantic herring (Winters et al., 1993) but has not, to our knowledge, been proven to occur within one stock. One report referring to Atlantic cod from the Norwegian coast (Kjesbu et al., 1996a) indicates that much of the variability in egg size occurs during the final maturation rather than variability in follicular size when final maturation occurs. Overall our view is that the relationship used for fecundity standardisation should be documented, including the follicle size reference point along with the unadjusted results.

Different image analysis configurations used by four institutes to collect the autodiametric calibration data produced a low CV of fecundity estimates for new predictions. The data can be accumulated without intervention (Thorsen and Kjesbu, 2001) in automatic mode and has utility for a number of species. Since it is an automatic process it is important that all follicular classes of interest are measured with equal selectivity, including cortical alveoli, atretic, or hydrated follicles. We suspect that the cause of the higher fecundity CV

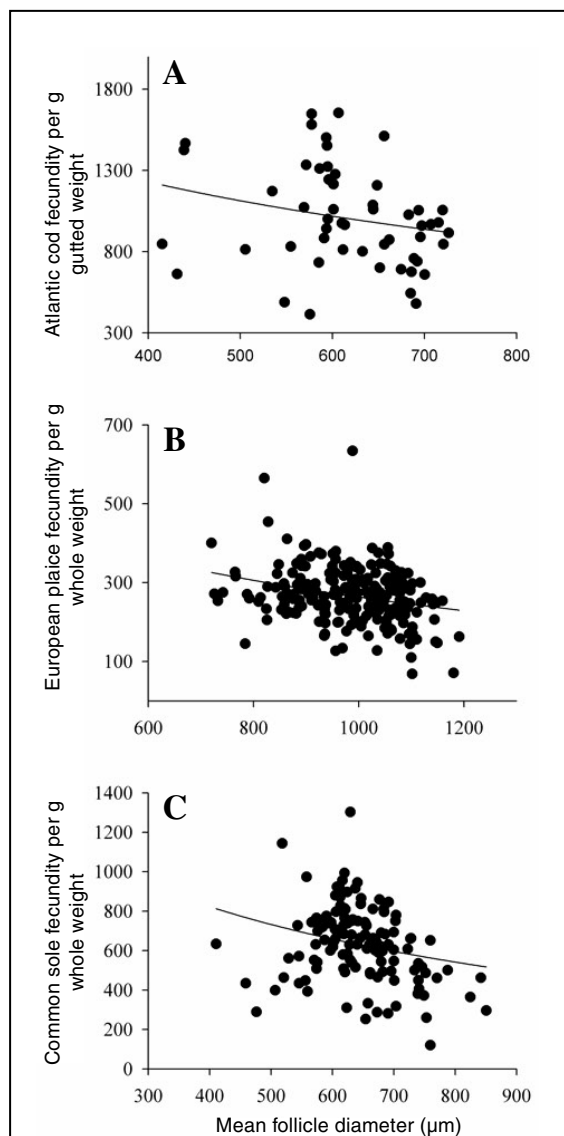


Figure 6

Reduction in relative fecundity (F_{bw}) during maturation, measured as mean follicle diameter (D_f , μm) in Atlantic cod (*Gadus morhua*), European plaice (*Pleuronectes platessa*), and common sole (*Solea solea*) (A–C, respectively) collected from the Irish Sea during 1995. The equation for the fitted line and regression coefficients are shown in Table 4.

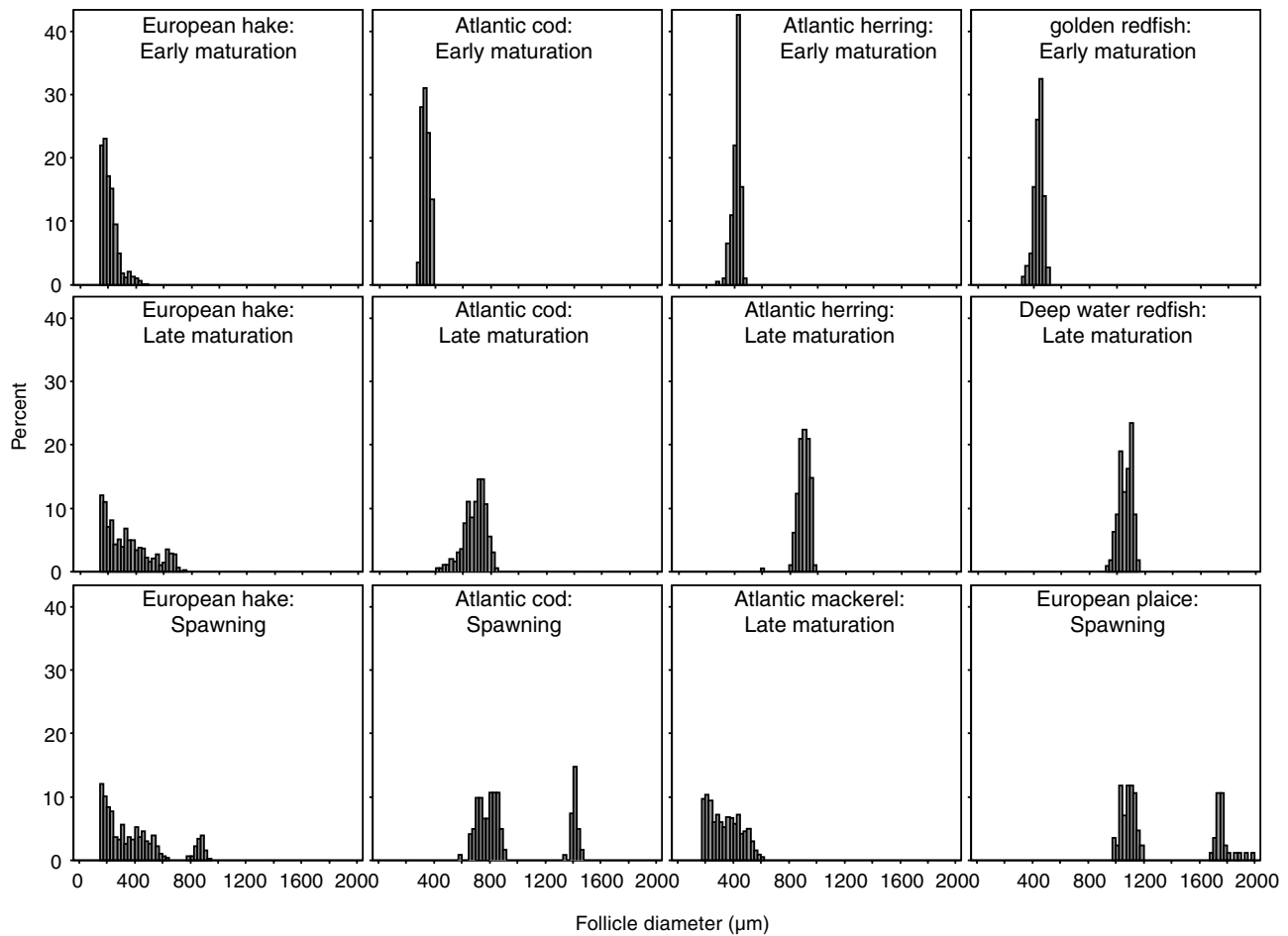


Figure 7

Follicle number per g of ovary (percentage of total count) per 25- μ m class interval follicle diameter found in Atlantic cod (*Gadus morhua*), European hake (*Merluccius merluccius*), Atlantic herring (*Clupea harengus*), Atlantic mackerel (*Scomber scombrus*), European plaice (*Pleuronectes platessa*), redfish (deep water redfish [*Sebastes mentella*] or golden redfish [*Sebastes marinus*]) used to produce the autodiametric calibrations (Table 5) by Institutes AZTI (A, stained rose bengal), Cefas (B, stained with periodic acid Schiff's, except European plaice with eosin), CSIC (C, hake rose bengal, redfish unstained), and IMR (D, unstained).

Table 4

Regression coefficients used to fit relative potential fecundity (F_{bw}) with mean follicle diameter (D_f , μ m) in the following equation $F_{bw} = a \times \ln(D_f) + b$ using data collected from Atlantic cod (*Gadus morhua*), European plaice (*Pleuronectes platessa*), and common sole (*Solea solea*) caught in the Irish Sea during 1995.

Species	Coefficient	SE	<i>t</i>	<i>P</i>	
Atlantic cod, <i>n</i> =54	b	4356	2039	4.6	<0.0001
	a	-521	317	-1.6	0.1068
European plaice, <i>n</i> =220	b	1578	318	55.0	<0.0001
	a	-190	46	-4.1	<0.0001
Common sole, <i>n</i> =129	b	3257	929	6.8	<0.0001
	a	-406	143	-2.8	0.0055

Table 5

Details of the autodiametric calibration relating follicle number (F_{ow}) to follicle diameter (D_f , μm) using a linear equation $\ln F_{ow} = a \times \ln D_f + b$ and polynomial equation (5) $\ln F_{ow} = a \times \ln D_f + b \times \ln D_f^2 + c$ fitted to data collected by each institute: AZTI, Cefas, CSIC, and IMR for each species: Atlantic cod (*Gadus morhua*), European hake (*Merluccius merluccius*), Atlantic herring (*Clupea harengus*), Atlantic mackerel (*Scomber scombrus*), European plaice (*Pleuronectes platessa*), and redfish (deep water redfish [*Sebastes mentella*] or golden redfish [*Sebastes marinus*]).

Species	Institute	a	b	c	N	r^2
Atlantic cod	Cefas	-3.106	28.777		28	0.972
Atlantic cod	Cefas polynomial	17.234	-1.5497	-37.864	28	0.986
Atlantic cod	IMR	-2.700	26.088		47	0.988
European hake	AZTI	-2.157	22.293		157	0.774
European hake	CSIC	-2.196	22.758		245	0.780
Atlantic herring	IMR	-2.718	26.287		23	0.971
Atlantic mackerel	Cefas	-2.528	25.030		78	0.761
European plaice	Cefas	-2.910	27.442		150	0.980
Redfish	CSIC	-2.551	25.040		147	0.948
General (excluding European hake)	All institutes except AZTI	-2.750	26.371		475	0.979

reported for species like European hake or Atlantic mackerel maybe attributed to the ovary being packed with a larger, and perhaps more variable partial volume of PVF associated with a continuous follicular distribution. Further analysis to determine the source of variation in the autodiametric calibration for fish with a continuous follicular frequency distribution is therefore considered worthwhile. Calibration data that included spawning Atlantic cod was best described by a polynomial model, although the additional term was not significant for the European plaice, even though the data included fish with hydrated follicles and POF. The difference may arise because European plaice produce fewer egg batches, about five (Urban, 1991), compared to between 14 and 21 in Atlantic cod (Kjesbu et al., 1996b). Thus, residual POFs in Atlantic cod ovaries should take up increasingly more space in the ovary towards the end of the spawning season changing the relative partial volume taken up by residual vitellogenic follicles.

An alternative to full automation is to use a semi-automatic analysis so that follicles that are not measured by the automatic analysis can still be measured manually. In practice, the dominant fecundity follicles were measured in automatic mode and then other follicular types, such as POFs or atretic follicles, are manually assigned and measured accumulating the measurements in user defined classes. This information can be used for more qualitative aspects, such as an overview of atresia intensity or confirming fish are at an advanced state of maturity, and also to provide a means to exclude fish that have started spawning. Our experience shows that POFs will arise from a synchronous ovulation that will produce a cohort of POFs of similar size and shape thus mak-

ing their identification more certain. In practice we keep a tally of identified POFs in a separate class and reject the fish from the fecundity data set to apply the annual egg production method if five or more POFs with similar structures are found. The hydrated cohort were split from the vitellogenic mode to determine the batch fecundity by inspection of the frequency distribution produced from the follicular measurements. This provides a further advantage for the study of batch fecundity because it is easier to see and separate the next batch compared to the traditional gravimetric method described previously (Hunter and Macewicz, 1985a).

In conclusion the present study has shown that image analysis and the autodiametric method have wider application than originally reported (Thorsen and Kjesbu, 2001; Klibansky and Juanes, 2008). Although one report (Friedland et al., 2005) indicated caution in this respect, the range of spawning strategies and institutes participating in this study indicate that for species with a discontinuous follicular frequency distribution, the method is also reliable. However, the authors have demonstrated that a calibration should be done to validate the method in all new applications whether it involves new species, equipment, or situation. The use of the pipette makes it possible to take quantitative fecundity samples in situations where accurate balances, measuring to an accuracy of 0.1 mg, will not function. In addition this provided a means to calibrate the autodiametric method for routine quality control and substantially reducing the use of toxic fixative. Substantial histology costs can be avoided by improving the interpretation of whole mounts and the approach has great utility to study the fate of fecundity during the spawning season.

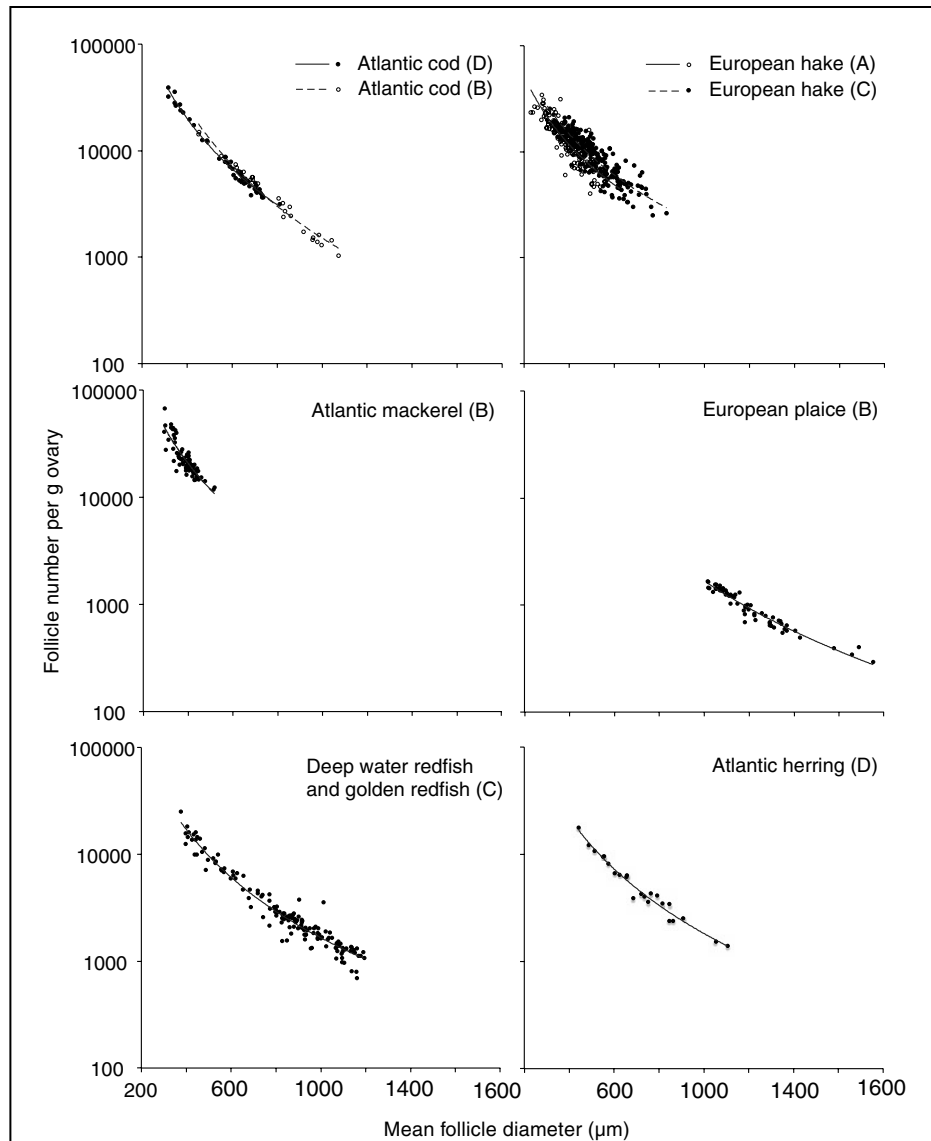


Figure 8

Autodiametric calibrations shown as scatter plots and fitted lines for Atlantic cod (*Gadus morhua*), European hake (*Merluccius merluccius*), Atlantic herring (*Clupea harengus*), Atlantic mackerel (*Scomber scombrus*), European plaice (*Pleuronectes platessa*), redfish (deep water redfish [*Sebastes mentella*] or golden redfish [*Sebastes marinus*]) used to produce the autodiametric calibrations (Table 5) by Institutes AZTI (A stained Rose Bengal), Cefas (B stained with periodic acid Schiff's, except European plaice with eosin), CSIC (C hake Rose Bengal, redfish unstained), and IMR (D unstained).

Acknowledgments

This study was jointly funded under European Union Frame Work V Q5RS-2002-01825 and the Institutes in England (Department of the Environment, Food, and Rural Affairs), Norway (Institute of Marine Research), and Spain (Consejo Superior de Investigaciones Científicas, and AZTI Tecnalia

(publication number 424)). The first author would like to acknowledge the contribution of J. Pilkington who wrote the software code for Myrmica. This software will be distributed at no charge under a Free Software Foundation License at www.myrmica.co.uk.

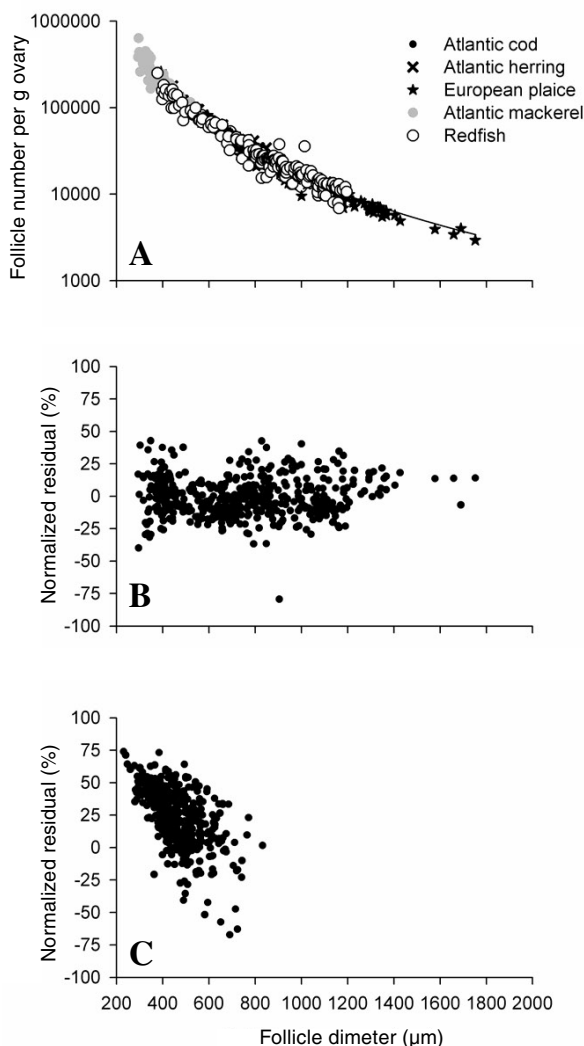


Figure 9

(A) General autodiometric calibration for Atlantic cod (*Gadus morhua*), Atlantic herring (*Clupea harengus*), Atlantic mackerel (*Scomber scombrus*), European plaice (*Pleuronectes platessa*), redfish (deep water redfish [*Sebastes mentella*]) or golden redfish (*Sebastes marinus*) used to produce the autodiometric calibrations (Table 5) by Institutes Cefas (B, stained with periodic acid Schiff's, except European plaice with eosin), CSIC (C, hake rose bengal, redfish unstained), and IMR (D, unstained) based on the combined data D_f and follicle number (F_{ow}). The parameters (Table 5) for the fitted line were based on Equation 4 ($\ln F_{ow} = a \times \ln D_f + b$). P for a and b was <0.001 . (B) Plot of normalised residual $(F - O_f) / F \times 100$ where O_f = observed value for Atlantic cod, Atlantic herring, Atlantic mackerel, European plaice, and redfish (deep water redfish (*Sebastes mentella*) or ocean perch (*Sebastes marinus*)) and F = fitted value based on F_{ow} against D_f (Eq. 4). (C) Plot of normalized residual $(F - O_h) / F \times 100$ where O_h = observed fecundity for European hake (*Merluccius merluccius*), and F = fitted fecundity value based on the observed F_d for hake substituted in the general model (Eq. 4).

Table 6

Predicted values (coefficient of variation) of follicle number per gram of ovary (F_{ow}) from mean follicle diameter (D_f , μm) using a linear equation $\ln F_{ow} = a \times \ln D_f + b$ and polynomial equation $^1 \ln F_{ow} = a \times \ln D_f + b \times \ln D_f + c$ fitted and regression parameters in Table 5 specific for each species (Atlantic cod [*Gadus morhua*], European hake [*Merluccius merluccius*], Atlantic herring [*Clupea harengus*], Atlantic mackerel [*Scomber scombrus*], European plaice [*Pleuronectes platessa*], redfish (deep water redfish [*Sebastes mentella*] or golden redfish [*Sebastes marinus*]) and institute (AZTI, Cefas, CSIC, IMR).

Species: Institutes	D_f values substituted in the linear and polynomial ¹ equations									
	300	450	600	750	900	1050	1200	1350	1500	1650
Atlantic cod: IMR	14665 (0.87)	6744 (0.94)	3692 (1.01)							
Atlantic cod: Cefas	18033 (1.57)	7380 (1.63)	3690 (1.75)	2094 (1.89)	1297 (2.07)					
Atlantic cod ¹ : Cefas	14488 (1.18)	7358 (1.11)	3819 (1.21)	1993 (1.31)	1061 (1.55)					
European hake: AZTI	21742 (2.20)	9065 (2.41)	4873 (2.62)	3011 (2.84)						
European hake: CSIC	27700 (2.20)	11368 (2.38)	6043 (2.56)	3684 (2.73)						
Atlantic herring: IMR	15990 (1.35)	7315 (1.38)	3988 (1.47)	2430 (1.60)	1598 (1.75)					
Atlantic mackerel: Cefas	15448 (1.82)									
European plaice: Cefas	15703 (1.73)	6798 (1.48)	3551 (1.59)	2089 (1.70)	1334 (1.81)	904 (1.91)	642 (2.02)	472 (2.13)	358 (2.2)	
Redfish: CSIC	12703 (1.97)	6097 (2.11)	3450 (2.25)	2166 (2.19)	1462 (2.52)	1040 (2.65)				

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