

Introduction

Estimation of individual egg production is a key step to understand the relationship between stock and recruitment or to assess spawning stock biomass using egg production. Here we demonstrate a fecundity method that uses less toxic fixative and is less labour intensive whilst automating the work and improving the accuracy of population fecundity estimates. We show:

- a solid displacement pipette (Figure 1) for fecundity sampling.
- if follicle size and density (gram⁻¹) is homogenous in the ovaries of *Gadus morhua* and *Merluccius merluccius*.
- the precision and utility of the Auto-diametric method (Thorsen & Kjesbu 2001) applied by 4 institutes (AZTI, Cefas, IIM-CSIC and IMR) to six species with different spawning strategies (Figure 2).
- an examination of whole mounts (disaggregated clumps of formaldehyde fixed follicles) replaces histology? The morphology of atretic and post ovulatory follicles are compared in each preparation.
- estimation of down regulation (production of atretic follicles) of potential fecundity during maturation without histology.

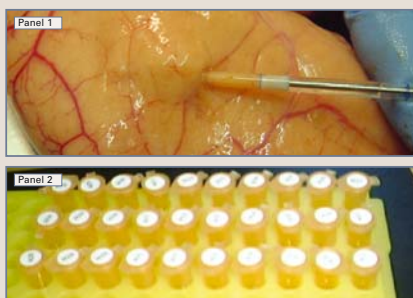


Figure 1: **Panel 1** Picture of the pipette removing 106 mg sample to blue line from a cod ovary. **Panel 2** Triplicate fecundity samples were stored in 2.5 ml plastic tubes fixed in 1.2 ml of 3.0% neutral buffered formaldehyde solution to reduce exposure and risk of spilling large quantities of formaldehyde during sampling.

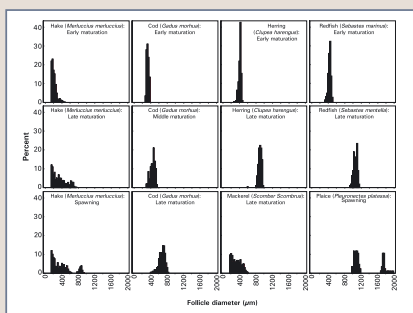


Figure 2: Follicle size frequency histograms taken from each species used in the study.

Methodology

Samples of ovary tissue were taken from each species using the gravimetric method (Hunter et al., 1989) from the whole ovary after fixation to calibrate the Auto-diametric method. In the case of cod a quantitative sample was also removed from the fresh ovary by pipette (Figure 1). Follicle size and number were measured by image analysis collecting the data with a digital camera connected to a PC running image processing software available at each institute. In each case a standard greyscale was used to prepare images of the follicles viewed under a binocular microscope. Ovary tissue preparations were viewed dispersed in water before and after staining by periodic acid Schiff's (PAS) followed by PAS Mallory staining after processing into histological resin sections.

Results

Fecundity determination

Estimates of fecundity and follicle diameter were significantly different ($P < 0.05$) depending on whether the ovary was fixed whole, to carry out gravimetric analysis, or in small tissue aliquots taken by the pipette. Samples taken from the anterior, middle and posterior of the left and right ovaries from 7 cod and 113 hake indicated that follicle density was not related to sampling location ($P > 0.05$). Mean follicle diameter ($P > 0.05$) was uniform across sampling locations in hake but differed, although not consistently, across sampling locations in cod (Figure 3). Nonetheless, it was possible to fit Auto-diametric models (Figure 4) relating follicle density to mean follicle diameter for all species.

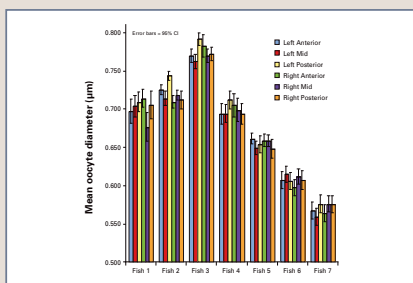


Figure 3: Mean follicle diameter recorded at the anterior, middle and posterior of the left and right ovaries in 7 cod (number of follicles measure in each sample varied between 164 and 200).

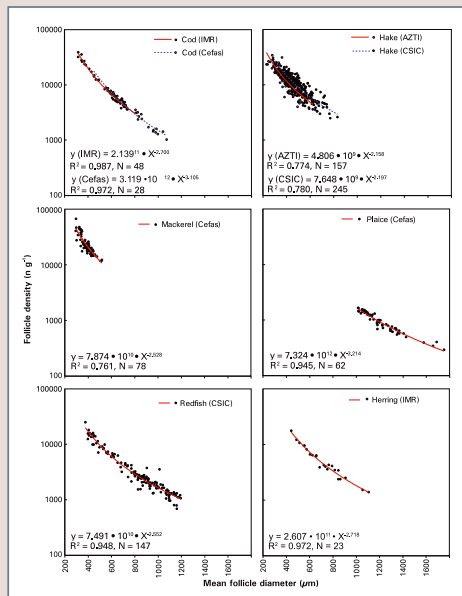


Figure 4: Plots showing the relationship between follicle density in the ovary and mean follicle diameter in 6 species examined (Figure 2).

Interpretation of whole mounts in relation to histology

The appearance of atretic follicles in cod is shown (Figure 5), both in whole mount and histological section. A comparison of atretic follicle counts prepared from the sample viewed as whole mount tissue and as a histological section showed close correspondence (Figure 6). Post ovulatory follicles (POFs) in cod could also be identified in whole mounts and their size and shape corresponded to their form seen in histological section (Figure 7). POFs shrink rapidly post ovulation but were found to persist over 4 months in cold 5°C water (data not shown) or a simulated North Sea summer warming temperature cycle. As spawning progresses small POFs become more abundant in the ovary making the classification of fish as pre-spawning or spawning increasingly more accurate.

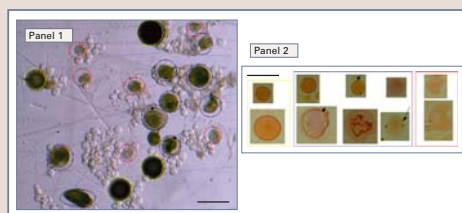


Figure 5: **Panel 1** Image taken from an unstained whole mount prepared from an ovary biopsy (9°C atresia experiment 2004) containing high levels of alpha (blue circles) and beta atretic follicles (red circles) and normal vitellogenic follicles (yellow circles). **Panel 2** Histological section of the biopsy in Panel 1 showing the same classes of follicle after processing into histological section. Arrows point to the disintegrating chorion used for classification. The scale bar = 500µm.

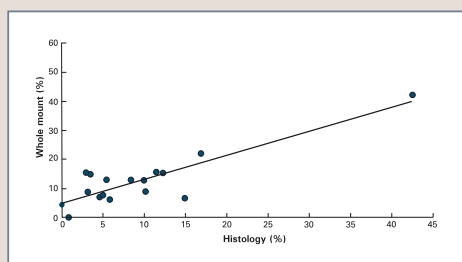


Figure 6: Comparison of atretic follicle counts made in whole mount (W) and histological section (H) The equation for the fitted line is $W=H \cdot 0.82 + 4.97$ ($n=18$, $r^2=0.75$).

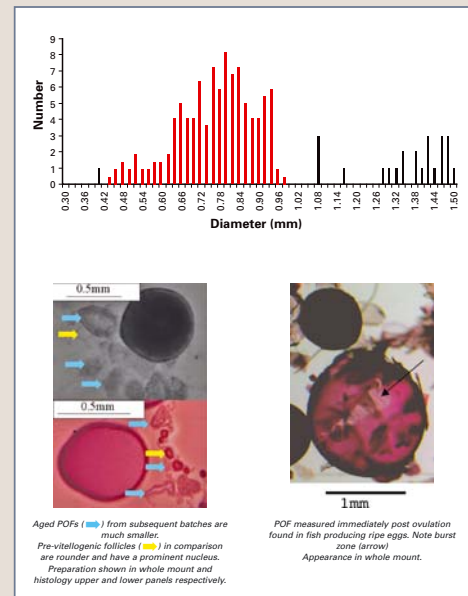


Figure 7: Histogram of whole mount ovary tissue sample showing maturing follicles (red bars) and POFs (black bars). Aged POFs (blue arrows) from subsequent batches are much smaller. Pre-vitellogenic follicles (blue arrows) in comparison are rounder and have a prominent nucleus. Preparation shown in whole mount and histology upper and lower panels respectively. POF measured immediately post ovulation found in fish producing ripe eggs. Note burst zone (arrow) Appearance in whole mount.

Down-regulation of potential fecundity during maturation

Relative fecundity of cod declined significantly (Figure 8) during maturation. This decline was fastest in the early stages, but close to zero when the leading cohort of follicles approaches final maturation. In the same samples follicular atresia (intensity or number of atretic follicles g⁻¹ female) was also present in some females (prevalence). At present there is sparse published data to convert the standing stock of atresia into daily rates of potential fecundity down regulation.

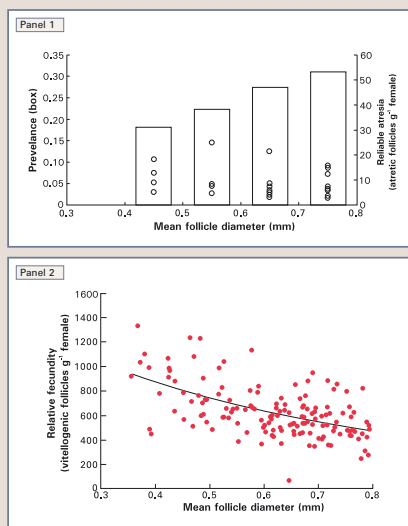


Figure 8: Reduction of relative fecundity (fecundity / total weight) in North and Irish Sea cod collected in 2003 and 2004. **Panel 1** shows the prevalence of atretic follicles (proportion of fish with atresia on the left axis and atresia intensity (atretic follicles g⁻¹ female whole weight)). **Panel 2** shows the decline in relative fecundity (Pg) in relation to the mean diameter (mm) of vitellogenic follicles (Fd) in the ovary. The fitted line was from the equation $Fg = a \ln Fd + b$, where $a = -581.9$ and $b = 340.2$, $n = 146$, $r^2 = 0.30$, P for both factors < 0.0001 .

Conclusions

The pipette will take quantitative samples of follicles from fresh, ripe ovaries that require only small quantities of fixative so reducing people's exposure to, and the quantity required of, fixative when working at sea. A small correction is required to fecundity determined with the pipette (in the case of cod in this experiment a raising factor of 1.06) compared to gravimetric samples. Follicle diameter was reduced when the ovary was fixed whole, but the Auto-diametric calibration takes this into account if the fixation method does not vary between the calibration and application.

The hake ovary was homogenous for both follicle density and size whilst, in contrast, the cod ovary was homogenous for follicle density but not for follicle size. Thus, we recommended that cod ovary sub-samples are taken from the left ovary from the rear and centre regions.

The Auto-diametric method has general utility and can be applied to several species to provide precise estimates of fecundity. A range of image analysis configurations can be used to acquire the follicle size data and the outputs can be used for determination of batch fecundity or maturation status.

Whole mounts can be interpreted to identify post ovulatory follicles and count atretic follicles thereby reducing the need for histology. The accuracy of potential fecundity will be improved if ovary samples are taken close to the time of spawning when the rate of down regulation is at its lowest, or be standardised by maturity status.

Acknowledgement

Defra and the European Commission through contract number 05R5-2002-01825

References

Hunter, J. R., B. J. Maciewicz, and C. A. Kimbrell. 1989. Fecundity and other aspects of the reproduction of sablefish, *Anoplopoma fimbria*, in central California waters. *Rep. Calif. 30*:61-72.

Thorsen, A., and O. S. Kjesbu. 2001. A rapid method for the estimation of oocyte size and potential fecundity in Atlantic cod using computer-aided particle analysis system. *Journal of Sea Research*. 46:295-308.