

Perspective Note

Quantification of ovarian condition in fish: a safer, more precise alternative to established methodology

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Abstract

The ability to accurately quantify ovarian condition in cultured species of fish is of paramount importance in the understanding of their reproductive biology and the development of appropriate culture conditions. Here, we discuss stereological analysis, a mathematically proven technique that determines 3-dimensional topology and geometry from 2-dimensional thin sections, as a safer and more precise replacement for existing methodology. © 2002 Ifremer/CNRS/Inra/IRD/Cemagref/Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

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Several techniques, with varying degrees of complexity, have been used to assess ovarian condition in teleosts. Early techniques addressed gross external appearance of the ovary, simple oocyte size distribution, basic classification of oocyte development by histological appearance, or the proportional estimation of oocyte developmental stages on histological sections. Over recent years, the most commonly used technique has been the digestion of ovarian tissue in a mercury-based solution (Gilson's Fluid). During digestion, oocytes become progressively separated from surrounding connective tissue. Oocyte size distribution is then assessed by volumetric sub-sampling from the digested sample followed by manual counting and sizing. Although informative and reasonably popular, this technique has several downfalls. Firstly, Gilson's Fluid is highly toxic (due to high mercury content) and complete digestion of ovarian tissue can take weeks to several months, depending upon the species concerned. Moreover, counting and sizing separated oocytes is difficult, time-consuming and potentially hazardous to the operator. Sub-sampling of separated oocytes is particularly difficult, even if oocytes are thoroughly mixed immediately prior to sub-sampling. Sinking rate is depen-

dent upon oocyte size, and stratification according to oocyte size is common, making the sub-sampling technique prone to error. Automated particle counters are sometimes used to aid counting and sizing but only operate over a pre-specified oocyte size threshold; pieces of connective tissue are also sometimes counted in error. Furthermore, fixation and histological processing can distort and shrink oocytes, thereby contributing to inaccuracy. This is particularly evident with Gilson's Fluid. A further major disadvantage is that it is impossible to assume that data obtained from this technique are strictly accurate as developmental classification of oocytes depends solely on oocyte size measurement. The development of dependable quantification techniques that do not utilise Gilson's Fluid is therefore of great importance to research into fish reproduction and the subsequent development of appropriate broodstock management regimes.

A major breakthrough is likely to occur as awareness of point counting stereology gains increasing attention from fisheries biologists. This technique, conceptualized in the 1960's, determines 3-dimensional geometric and topological structural information of a specimen from flat histological sections. This involves the use of mathematical formulae to define quantitative relationships between various features of flat sections and their source in original space. Methodology involves placing a test system (e.g. grid points) over

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planes cut through a specimen. Since the test system has known properties, we can derive information about the unknown properties of our specimens by observing how they interact with the known test system. Stereological methods have become widely used morphometric techniques in metallurgy, geology, and medicine but have been scarcely used in fish physiology. Recent stereological studies involving the estimation of fecundity in marine fish (Emerson et al., 1990) and the gonadal histomorphology of Brazilian sardine *Sardinella brasiliensis* (Isaac-Nahum et al., 1988), gudgeon *Gobio gobio* (Kestemont, 1987, 1990), Argentinian sand perch *Pseudoperca semifasciata* (Macchi et al., 1995), tinfoil barb *Puntius schwanenfeldii* (McAdam et al., 1999), tilapia (Srisakultiew, 1993; Coward and Bromage, 1998; Coward and Bromage, 1999ab, 2001) and bluefin tuna *Thunnus thynnus* (Medina et al., 2002) are only just beginning to raise awareness of this powerful technology.

Precise methodology is beyond the remit of the present paper but is discussed at length by Weibel (1979) and Elias and Hyde (1983). Stereology is based upon the “Delesse principle” that considers an object (e.g. ovary) placed into an x, y, z coordinate system and cut into thin sections parallel to the x, z plane. In this scenario, the proportional area occupied by a given particle of interest (A_{po} , a discrete oocyte developmental stage for example) is equal to the total particle sectional area (A_{so} , the total area occupied by the given particle within a given thin section) divided by the total area of the section (A_{st}); $A_{po} = A_{so}/A_{st}$. Estimates of the proportional area of a given oocyte stage (A_{po}) can hence be obtained using a relatively small number of grid-points super-imposed over the thin section of interest. The estimated proportional area (P_{po}) occupied by an oocyte stage can therefore be readily estimated ($P_{po} = P_{so}/P_{st}$) from histological sections using a random grid sampling procedure (see Weibel, 1979; Elias and Hyde, 1983) and a microscope equipped with an eyepiece graticule grid. Approximately 300 grid points, spread over a number of independent sections and fields, is required to establish an acceptable variance (Weibel, 1979). This number might, however, be difficult to achieve for the more advanced stages of oocyte development because of their much larger size relative to earlier stages. Mathematics proves that the area occupied by a given particle is equal to its volume ($V_{po} = A_{po} = P_{po}$) assuming a section of zero thickness (see Weibel, 1979; Elias and Hyde, 1983). The proportional volume fraction (expressed as %) occupied by each oocyte stage can therefore be determined in a simple manner.

Volume fraction, although an informative physiological parameter itself, can subsequently be used to determine the relative numbers of oocyte developmental stages per unit volume of ovary. These figures can be extrapolated to represent total ovarian volume and hence produce data comparable to that derived from Gilson analysis. In brief, volume fraction data for each oocyte stage are corrected for oocyte size (K) and shape (β) distribution. The number of

stage ‘x’ oocytes in a unit volume (N_{vx}) can thus be determined ($N_{vx} = K_x/\beta_x \cdot N_{ax}^{3/2}/V_{ix}^{1/2}$, where N_{ax} = number of stage ‘x’ per unit area and V_{ix} = proportional volume fraction occupied by oocyte stage ‘x’). See Weibel and Gomez (1962), Elias and Hyde (1983) and Emerson et al. (1990) for detailed descriptions of the methodology required.

Validative comparisons of quantitative ovarian histology between Gilson Fluid digestion and stereological methods have, thus far, been undertaken in a very limited number of fish. In the Nile tilapia *Oreochromis niloticus*, Srisakultiew (1993) found no statistical difference between the two methods. Similar results were obtained for the red-bellied tilapia *Tilapia zillii* (Coward and Bromage, unpublished), although estimates of the numbers of atretic oocytes were significantly underestimated by the Gilson method in one of our experiments. Emerson et al. (1990) reported no significant differences between the two methods for sole *Solea solea* and herring *Clupea harengus*, but in the case of Atlantic mackerel *Scomber scombrus* (L.), stereology produced significantly higher estimates of fecundity. These apparent discrepancies are cause for concern and highlight the need for further validating studies. The main source of error appears to be down to the fact that no histological assessment is made of oocytes in the Gilson technique; analysis relies solely upon oocyte size distribution. Histological criteria must be considered when classifying an individual oocyte into a discrete developmental category, particularly in the case of those undergoing degenerative processes such as atresia. Gilson analysis is highly likely, therefore, to underestimate the numbers of atretic oocytes, particularly those in the very early stages of degeneration. A major advantage of stereology, therefore, is that it classifies discrete oocyte development stages by histological criteria and not merely oocyte size, thereby allowing far more accurate quantification of the distribution of different developmental stages. We must also consider that oocytes of the same size may not necessarily be in the same stage of development and that even within an individual oocyte there might be periods when some growth phases overlap. Degenerative stages such as atretic oocytes and post-ovulatory follicles are inherently difficult to quantify by histomorphometry due to wide fluctuation in size and shape; these are factors that limit the effectiveness of both stereology and the Gilson method. On the other hand, however, early degeneration can be determined histologically in oocytes that have not yet begun to alter in size and shape, thereby allowing stereology to determine relative proportions of early degenerative stages. Once oocyte size and shape have been compromised, stereological analysis becomes redundant.

Consequently, we suggest that point-counting stereological methods can be used as more accurate and much safer alternatives to the more established Gilson’s Fluid technique in at least some fish species. Rigorous evaluation is, however, recommended before the technique is routinely

applied to other species. Furthermore, although stereology has huge potential for fisheries research, the methodology can be rather time-consuming and the underlying mathematical theory foreboding at best. However, basic point counting should become much easier as digital imaging systems become more readily available thereby removing the need for intricate knowledge of the associated mathematics. Calculations can be simplified by using carefully designed computer spreadsheets or dedicated image analysers. A further important consideration is that to avoid gross over-estimation, stereology must rely heavily upon data collection from highly independent sources. Achieving true independence over a large number of graticule grid points may not be easy in some species, particularly those with large egg size. To avoid this, the selected method requires rigorous experimental design and testing. Since no underlying assumptions are made about the specimen structure however, a major advantage of stereology is that the method remains statistically unbiased. Reliance upon systematic, uniform, and random specimen sampling yields accurate estimates of the characteristic being sampled. Care also needs to be taken to ensure that raw data are treated in the appropriate manner. All volume fraction data (ultimately expressed by stereology equations as %) should be converted to proportions and ARCSine transformed prior to statistical analysis to avoid skew and truncation. It is important to understand that stereology does not predict exact quantities of a specimen feature, rather, stereology encompasses a suite of methods and mathematics for the accurate estimation of the mean value of a quantity and its standard deviation.

In summary, stereology is recommended as a safer and more precise substitute for the determination of ovarian histomorphometry in teleosts than the more established Gilson Fluid digestion. Furthermore, stereology is particularly useful when used to correlate ovarian dynamics to other physiological parameters (e.g. endocrine, nutritional, behavioural and environmental status) (e.g. Coward and Bromage, 1998, 1999a). Stereological methods have also proved suitable for the analysis of tissue samples obtained by intra-ovarian biopsy (Coward and Bromage, 2001); small biopsies taken from the ovaries of anaesthetised fish can yield highly quantitative information on gross ovarian condition and thereby avoids the need for sacrifice. As publicity and awareness of stereology grows within the field of fish physiology, and as sophisticated digital imaging

systems become more readily available, the methods introduced herein should prove to be an immensely powerful and elegant tool at the fish physiologists disposal.

References

- Coward, K., Bromage, N.R., 1998. Histological classification of oocyte growth and the dynamics of ovarian recrudescence in *Tilapia zillii*. *J. Fish Biol.* 53, 285–302.
- Coward, K., Bromage, N.R., 1999a. Influence of conspecific contact upon blood sex steroid levels and the dynamics of ovarian development in female red-bellied tilapia, *Tilapia zillii*. *J. Appl. Aquac.* 9, 23–34.
- Coward, K., Bromage, N.R., 1999b. Spawning frequency, fecundity, egg size and ovarian histology in groups of *Tilapia zillii* maintained upon two distinct food ration sizes from first-feeding to sexual maturity. *Aquat. Living Resour.* 12, 11–22.
- Coward, K., Bromage, N.R., 2001. Stereological validation of ovarian biopsy as a means of investigating ovarian condition in broodstock tilapia in vivo. *Aquaculture* 195, 183–188.
- Elias, H., Hyde, D.M., 1983. A guide to practical stereology. Karger, USA.
- Emerson, L.S., Greer Walker, M., Witthames, P.R., 1990. A stereological method for estimating fecundity. *J. Fish Biol.* 36, 721–730.
- Isaac-Nahum, V.J., Cardoso de D., R., Servo, G., Rossi-Wongtschowski del B., C.L., 1988. Aspects of the spawning biology of the Brazilian sardine, *Sardinella brasiliensis* (Steindachner, 1789) (Clupeidae). *J. Fish Biol.* 32, 383–396.
- Kestemont, P., 1987. Reproductive cycle of the gudgeon *Gobio gobio* L. 1. Seasonal variations in the histology of the ovary. *J. Appl. Ichthyol.* 3, 145–157.
- Kestemont, P., 1990. Dynamic aspects of ovogenesis in an asynchronous fish, the gudgeon, *Gobio gobio* (L.) (Teleostei, Cyprinidae), under controlled temperature and photoperiod conditions. *Aquat. Living Resour.* 3, 61–74.
- Macchi, G.J., Elias, I., Burgos, G.E., 1995. Histological observations on the reproductive cycle of the Argentinian sandperch, *Pseudoperca semifasciata* (Osteichthyes, Pinguipedidae). *Scient. Mar.* 59, 119–127.
- McAdam, D.S.O., Liley, N.R., Tan, E.S.P., 1999. Comparison of reproductive indicators and analysis of the reproductive seasonality of the tinfoil barb, *Puntius schwanefeldii*, in the Perak River, Malaysia. *Env. Biol. Fish.* 55, 369–380.
- Medina, A., Abascal, F.J., Megina, C., Garcia, A., 2002. Stereological assessment of the reproductive status of female Atlantic northern bluefin tuna during migration to Mediterranean spawning grounds through the Strait of Gibraltar. *J. Fish Biol.* 60, 203–217.
- Srisakultiew, P., 1993. Studies on the reproductive biology of *Oreochromis niloticus* (L.). Ph.D. thesis. Institute of Aquaculture, University of Stirling, Stirling, Scotland.
- Weibel, E.R., 1979. Stereological Methods. Vol. 1: Practical Methods for Biological Morphometry. Academic Press, London, U.K.
- Weibel, E.R., Gomez, D.M., 1962. A principle for counting tissue structures on random sections. *J. Appl. Physiol.* 17, 343.