

A key issue in the estimation of fecundity of oviparous fishes is whether or not the annual fecundity can be estimated from the standing stock of advanced oocytes in the ovary prior to the onset of the reproductive season. In some boreal species, frequently called total or isochronal spawners, all the eggs to be released in a season develop synchronously prior to spawning (hence the term isochronal) and spawning typically takes place over a short period (Holden and Raitt 1974). In such species, the standing stock of oocytes within a certain range of maturity classes is considered to represent the annual fecundity of the spawner. The groups of oocytes to be spawned in the season are usually identifiable because a distinct hiatus in oocyte maturity classes exists between the small, immature, unyielded oocytes that occur the year around and the synchronously maturing annual batch (Hickling and Rutenberg 1936; Yamamoto 1956). Although some of these fishes may spawn repeatedly during the season, for example, whiting and haddock, the standing stock of yielded eggs is considered representative of the annual fecundity (Hislop 1975; Hislop et al. 1978; Hislop, pers. commun.). An exception to this occurs when unfavorable conditions result in resorption of some of the advanced eggs in the ovary at the end of the season. The extent of this potential bias (overestimation of annual fecundity) is unknown. In many temperate and tropical fishes (frequently called multiple, partial, serial, or heterochronal spawners), annual fecundity is seasonally indeterminate and batch fecundity is the only useful measurement. In such fishes the standing stock of yielded eggs, regardless of maturity state, give no indication of annual fecundity because these fishes continuously mature new spawning batches throughout a typically protracted spawning season. In the active ovaries of fishes with indeterminate annual fecundity, the oocytes usually occur in nearly all maturity stages; they range in size continuously from small unyielded oocytes to yielded oocytes 0.4–0.7 mm diam., and no large hiatus exists between maturity classes of oocytes except for one between hydrated oocytes and advanced yielded oocytes which is of a temporary nature. Such fishes usually spawn many times during a season. The northern anchovy spawns at 7–10 d intervals for 2 or 3 mo and averages 20 spawnings per yr (Hunter and Boning 1981). and the scianid, *Seriphus politus*, has a similar reproductive output (DeMartini and Fountain 1981). Thus, for these fishes, identification of a predetermined annual spawning batch is a hopeless exercise, and the only useful fecundity measurement is the number of eggs produced in a single spawning batch (batch fecundity); annual fecundity is a function of both the batch fecundity and the number of spawnings per year. Spawnings are so numerous in these fishes that small unyielded oocytes would have to mature in a season to account for the number of spawnings (Hunter and Leong 1981). The standing stock of oocytes is occasionally used to estimate annual fecundity in such common fishes as *Scomber*, *Trachurus*, and *Merluccius*, which by the standard criteria have indeterminate fecundity. That annual fecundity is predetermined in such fishes is an assumption with little or no supporting evidence. The criteria and approaches for distinguishing between determinate and indeterminate fecundity are discussed in greater detail (Hunter and Macewicz 1985). The objective of this paper is to describe the methodologies for estimating batch fecundity in fishes with indeterminate seasonal fecundity. We do not consider the well documented methodology 'John R. G. Hislop, DAFS Marine Lab., P.O Box 101, Victoria Road, Aberdeen AB9 8DB. Scotland, pers. commun. Oct. 25, 1983 67 for fecundity estimation of fishes with seasonally determinate fecundity (see, for example, Holden

and Kaitt 1974). In anchovy and other fishes with indeterminate annual fecundity, the oocytes in active ovaries are typically distributed in 1–2 modes (Fig. 1), each mode representing a single spawning batch. Maturation of oocytes and vitellogenesis are a continuous cycle. When one spawning batch is spawned, another spawning batch is ready for the last stages of maturation and spawning (Fig. 2). Vitellogenesis proceeds rapidly after a spawning, with the ovary doubling in dry weight during the interval between spawnings (Hunter and Leong 1981). The final stage of maturation, hydration, is characterized by a rapid secretion of fluid of low specific gravity into the advanced eggs by the granulosa cells of the follicle (Fulton 1898). This fluid causes more or less complete fusion or solution of the yolk granules producing the translucent appearance of hydrated eggs. The volume of the egg or wet weight increases three- or four-fold (Fulton 1898), but the increase in dry weight is negligible (LeClus 1979a). In northern anchovy, hydration begins about 12 h before spawning when the eggs are between 0.6 and 0.8 mm (major egg axis) and causes a four-fold increase in wet weight of the ovary as the egg increases to 1.3 mm (major axis) (Hunter and Macewicz 1980; Hunter and Leong 1981) (Fig. 2). Ovulation and spawning soon follow completion of hydration in most clupeoids (anchovy, pilchard, sardines, and others) but in herring, a total spawner, ovulated eggs may be retained in the ovary for an extended period.

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HERRING YINIE S P A I K D EGG SIZE (mm) Figure 1.—Frequency distribution of oocyte diameter in the ovaries of herring, pilchard (Hidding and Rutenbergl936), and northern anchovy (Hunter and Leong 1981). Herring spawns single batch each year; other species are multiple-batch spawners. In the anchovy, the solid line shows a recently spawned female, the broken line a female about to spawn (just before hydration of the oocytes). (From Hunter and Leong 1982.)

SPAWN SPAWN R I I I I I I I I I 1 4 5 6 0 1 2 3 4
5 6 0 ELAPSED TIME FROM SPAWNING (day) Figure 2.—Maturation cycle of a 0.5 g northern anchovy female during peak spawning months where the average interval between spawnings is 7 d. The change in mean wet weight of the ovary is indicated on the left axis and the mean diameter of the oocytes in the most advanced spawning batch on the right axis. Data from Hunter and Goldberg (1980) and Hunter and Macewicz (1980).

68 LABORATORY PROCEDURES Identifying and Counting Oocytes in a Batch

A number of criteria have been used to identify the oocytes to be included in a spawning batch. These include 1) counts of all yolked oocytes; 2) estimation of the number of oocytes in the most advanced spawning batch by measuring the size distribution of oocytes in the ovary and identifying the most advanced (largest) modal group of oocytes; and 3) estimation of the number of oocytes in a spawning batch by counting the number of hydrated oocytes. The first method can be rejected because the standing stock of yolked oocytes gives indication neither of total fecundity nor of batch size in fishes with indeterminate fecundity and is appropriate only for fishes with determinate seasonal fecundity. The second method (oocyte size frequency) usually gives results similar to those based on counts of hydrated oocytes if females with highly advanced oocytes are used (Hunter and Goldberg 1980; Laroche and Richardson 1980). We believe the third method, counting the hydrated oocytes, is preferable because it requires less time and avoids the problem of partitioning oocytes between the most advanced mode and the adjacent group of smaller oocytes. Before describing methods 2 and 3, it is important to consider methods of sampling the ovary.

Methods of Sampling an Ovary

It is impractical to count and measure all advanced oocytes, or to count all hydrated oocytes in an

ovary, owing to the great fecundity of most marine fishes. Thus, regardless of the method used for identifying the spawning batch, ovarian subsamples are required, and these are related to either the ovarian weight (gravimetric method) or the total volume of an aqueous suspension of all oocytes in the ovary (volumetric method). The gravimetric method is based on counting oocytes in weighed samples of ovarian tissue and relating the tissue samples to the total ovary weight. In the volumetric method, the ovary is preserved in Gilson's fluid which frees the oocytes from the ovarian tissue by breaking down the connective tissue (see Baganel 1967 for the recipe for Gilson's fluid). The released oocytes are cleaned, put in a volumetric cylinder filled to a known volume with water, shaken to provide thorough mixing, subsamples of known volume are withdrawn using a Stempel pipette, and the oocytes staged and counted (Holden and Raitt 1974). Automatic oocyte counters may also be employed. The volumetric method may be used for batch fecundity estimation if the eggs constituting the batch are identified using the egg size-frequency method; however, the volumetric technique is inappropriate if the hydrated oocyte method is used because Gilson's fluid destroys hydrated eggs. Substantial shrinkage of oocytes occurs when ovaries are preserved in Gilson's fluid; an average shrinkage of 24% (compared to formalin-preserved ovaries) occurs when skipjack and yellowfin tuna ovaries are preserved in Gilson's fluid, but no differential shrinkage occurs among oocyte size classes (Joseph 1963). Thus, to make oocyte size classes comparable to live, or formalin-preserved, or histological sections, the extent of shrinkage must be measured and the data corrected. Treatment with Gilson's fluid also destroys the ovary, making histological analysis impossible. We use the gravimetric method of MacGregor (1957), which is somewhat similar to the gravimetric method "B" of LeClus (1977) who evaluated two gravimetric and one volumetric techniques. Although LeClus obtained very low coefficients of variation for all techniques in her 1977 methods paper, the relative fecundity estimated for the South African anchovy in a subsequent paper (LeClus 1979b) was as variable as those employing less complicated procedures (MacGregor 1957, 1968; Hunter and Goldberg 1980). Her use of vacuum dry weight of the ovary, instead of formalin wet weight, seems an unnecessary refinement. Natural variability in batch fecundity appears to be much greater than the variation caused by differences in the technique of sampling the ovary. The step-by-step procedure for the hydrated oocyte method is outlined below. It will be elementary to many biologists, but it is intended as a guide for inexperienced staff. Except for some details outlined in a subsequent section, the same procedure can be used to estimate batch fecundity using the oocyte size-frequency method.

The Hydrated Oocyte Method

1. The basic method is as follows: Numbers of hydrated oocytes in weighed tissue samples of formalin-preserved ovary are counted and the counts are then projected to estimate numbers of hydrated oocytes in the entire ovary which is assumed to be equivalent to batch fecundity.

2. Prior to batch fecundity estimation, females are accurately weighed, and the ovary removed, weighed, and stored in an individual vial of buffered 10% formalin. Ovaries which appear hydrated are noted along with an estimate of the numbers of free eggs in a collection jar (Hunter 1985). Only hydrated ovaries which have not lost oocytes are used for fecundity estimation; ovaries that have lost oocytes in the jar are rejected after histological examination because they contain postovulatory follicles.

3. Needed supplies and equipment include a balance sensitive to 0.1 mg (which should be checked with standard weights), a dissection microscope with a 10x objective, hand counter,

forceps, scalpel, bottle of glycerin (33% glycerol solution by volume) with eyedrop– per, glass slides (25 X75 mm), cover slips (22x50 mm), paper towels for blotting, and weighing paper. 4. Remove ovary from the formalin fixative and blot dry with paper towel. Break the ovarian membrane and remove three tissue samples of the ovary. The ovary is soft and the sample can be removed easily with the tip of the forceps or scalpel. Remove samples from posi– tions about one–third of the distance from each end of the ovary to insure that no two samples come from the same portion of the ovary (only one ovary, left or right, need be used). Try to obtain a tissue weight of 30–50 mg, as this will contain an adequate number of hydrated oocytes (100–200). Place sample on a preweighed piece of weighing paper and record weight to nearest 0.1 mg. Pieces of ovary can be added or removed to vary sample weight. 5. Place the sample on a slide and cover with 3 4 drops of glycerin. After 10–15 min, loosen the oocytes by gently tapping the piece of ovary with the blunt tip of the forceps. After the oocytes are loosened, add 3 or 4 more drops of glycerin, spread the sample over the slide, and cover with a cover slip so that it floats on the fluid. (We found that this concentration of glycerin had no effect on the diameters of oocytes taken from formalin–preserved ovaries, even after 24 h.) 6. Place the slide under the microscope, and with a hand counter tally the number of hydrated oocytes in the sample. Hydrated oocytes can be distinguished easily from other oocytes by their large size 69(usually 20.8 mm in the major axis in northern anchovy), wrinkled appearance when formalin preserved (jolked but nonhydrated oocytes usually retain their smooth surface contour), and by their trans– lucence (nonhydrated eggs are relatively opaque, Fig. 3). Some damage to the hydrated oocytes may occur during slide preparation. In some cases, the chorion may be ruptured and the yolk extruded. Do not count empty chorions, and count only those fragments judged to be major portions of the oocytes. 7. Batch fecundity (0for each female is calculated from the prod– uct of the number of hydrated oocytes (eggs) per unit weight in the tissue sample and the ovary weight (left and right sides combined) (2). 8. The egg production method requires that batch fecundity be expressed as a function of female weight and not length, i.e., $EY = f(w)$ where female weight (w) is the formalin wet weight of the female without the ovary (formalin wet weight can be converted to live weight using coefficients given in Hunter 1985). We use ovary–free wet weight of females since females with hydrated ovaries temporarily have a higher weight than the average female because of the increased weight of the hydrated ovary. The ratio of female body weight with– out ovary to female weight with ovary (excluding females with hydrated or immature ovaries) can be used to convert ovary–free wet weight to total body weight. This ratio in northern anchovy was 0.95 for female anchovy taken 1978–79 (Hunter and Macewicz 1980). Oocyte Size–Frequency Method If the number of females with hydrated oocytes is insufficient for a batch fecundity estimate, the more time–consuming oocyte size– frequency distribution method can be employed (MacGregor 1957). This method takes 1–3 h per fish (3 tissue samples per fish) as com– pared with 1–1.5 h for the hydrated oocyte method. In this method, a size– frequency distribution of oocytes is constructed and the most advanced modal group of oocyte size classes (the mode composed of the largest oocytes) is determined by inspection. The total number of oocytes within the oocyte size classes that constitute the advanced modal group is considered to be the spawning batch. This method usually gives results similar to those based on counts of hydrated oocytes if females with highly advanced oocytes are used (Hunter and Goldberg 1980; Laroche and Richardson

1980). The mean size of the group of oocytes that constitutes the most advanced spawning batch should be >0.5 mm, as estimates of batch fecundity are somewhat inflated if a less mature ovary is used (Hunter and Goldberg 1980). In northern anchovy, a tissue sample weight of 10 mg will insure that about 100 oocytes are included in the most advanced modal group of oocytes. All oocytes >0.3 mm in a tissue sample are counted using a set of hand counters and measured to the nearest 0.05 mm. We use an optical comparator at 50X magnification and measure the oocytes with a rule on the viewing screen of the comparator. A starting oocyte size of 0.3 mm is recommended to insure that a Figure 3.—Hydrated oocytes in a tissue sample taken from a northern anchovy ovary preserved in formaldehyde solution. A. Hydrated oocytes; B. Unyolked and yolked oocytes (before hydration); C Empty chorion of a hydrated oocyte (the chorion, or a major fragment of it, would be included in the count of hydrated oocytes for the hatch fecundity estimate). These hydrated oocytes were 1.2 mm long in the major axis. 70 sufficient number of 0.05-mm oocyte size classes exist below the most advanced modal group. Inclusion of these small oocyte size classes insures an accurate separation of the tail of the advanced mode of oocytes from the smaller oocytes adjacent to it. To separate the tail of the advanced modal group from the adjacent group of smaller oocytes, we use probability paper analysis (Harding 1949; Cassie 1954). In other respects, the oocyte size-frequency method is the same as the hydrated oocyte method, and the previous section can be used as a guide.

ACCURACY AND PRECISION

In this section we develop methods to evaluate the accuracy and precision of estimating batch fecundity using the hydrated egg methodology, and apply these procedures to northern anchovy fecundity data. Accurate estimation of batch fecundity depends upon selection of an unbiased location for the samples of ovarian tissue and the selection of an appropriate regression model to express the relationship between female weight and batch fecundity. The precision of the estimate depends upon the number of ovarian tissue samples taken per female and the total number of females. The weight of the individual tissue sample also affects precision, but we have not considered this element. We have instead kept the tissue samples within a weight range that yields about 100–200 hydrated oocytes per sample. In the first subsection we use analysis of variance to detect the possible effects of location of tissue samples within the ovary. In this analysis we use a sample of 12 northern anchovy ovaries in which 6 tissue samples were taken per ovary at specified locations. In the next subsection we determine how the number of tissue samples affect the precision of the fecundity estimate; consider various fecundity-weight models; and determine the optimum numbers of fish and tissue samples for a given cost. For this analysis we add an additional 12 fish to the sample used in the first section, all of which had 6 tissue samples per fish. In the third subsection we use seven data sets on anchovy batch fecundity taken 1951–60 and 1978–84 (n ranges, 19–127) to validate selection of the fecundity–female weight model and to assess the precision of regression estimates of batch fecundity. In the final subsections we consider how the number of fish in the sample affects precision of the fecundity estimate and how the batch fecundity varies among years.

Location of Tissue Samples Within the Ovary

To determine if location of the tissue samples affects estimates of batch fecundity in anchovy, we took 6 ovarian tissue samples from each of the 12 ovaries; three samples were taken from the left ovary and three from the right. In each set of three, one sample was taken in the center and the other two were about one-third of the distance from each end of the

ovary. The number of eggs per unit weight of the ovary (x) was calculated for each tissue sample. We tested effects of right or left ovarian side, and position of tissue samples within a side, using the two-way analysis of variance. The natural logarithm of x was used in the analysis because there is a positive correlation between the same sample mean and its standard deviation. The assumption of homogeneity within sample variance is violated when the means differ. No difference existed either in the location of the tissue sample within right or left sides of the anchovy ovary or between right and left sides (Table 1). Thus in northern anchovy, tissue samples can be taken from any location or from either the right or left sides. It should be noted, however, that all of our samples were of females taken at a time of day (1900–0100 h) when hydration was nearly complete. If females are taken earlier in the day, position effects may be likely because hydration does not proceed at a uniform rate throughout the ovary; rather it begins at the periphery and spreads to the central section of the ovary, producing larger hydrated oocytes (more mature) at the periphery than in the center. Thus the number of hydrated oocytes per gram of ovary may be higher in the central section and lower on the periphery at early stages of hydration. Variation in extent of hydration does not appear to be a major source of error, but it should be evaluated prior to making a fecundity estimate in a new species or when samples are taken at a new time of day.

Optimum Number of Tissue Samples and Numbers of Fish for Fecundity Estimation In this section we develop equations to estimate the optimum number of fish and tissue samples to be used for fecundity estimation using data on the northern anchovy. The precision of the sample variance (or mean square error) around the regression of batch fecundity on female weight (W) (a measure of the goodness of fit of the fecundity–weight model) was used to determine the optimum number of tissue and fish samples. This procedure required a definition of a general fecundity model and development of functions to express the error terms.

A. The general fecundity model—The true batch fecundity (Y) where all eggs are counted, and the fish weight relationship (w) is defined as: $Y = f(w) + A$ (1) where the error term (A) has a mean = 0, and variance = 0.1. Y and Y_j are interchangeable in later sections. Since all the hydrated eggs in a batch (Y) are not counted, $f(w)$ are fitted to the estimated batch fecundities (f_i calculated from m ovarian tissue samples per fish). Let's denote for the i th fish, $i = 1, \dots, n$: w_i = gonad-free fish weight = fish weight minus gonad weight Y_i = total number of hydrated eggs in the ovary y_{ij} = hydrated egg count in the j th tissue sample $j = 1, \dots, m$ z_j = weight of j th tissue sample Z_i = formalin wet weight of gonad m = number of tissue samples from an ovary M = maximum number of tissue samples in an ovary f_{est} = estimated total

number hydrated eggs in the ovary from the f_j = estimated total number of hydrated eggs in the ovary = $\sum_{j=1}^m y_{tj} / Z$, $m_j = 1, \dots, m$, = sample mean number of hydrated eggs = $\sum_{i=1}^n f_{ij} / n$ = estimate of batch fecundity from the regression model. Suppose t_j is an unbiased estimate of Y_j , then $t_j - Y_j + t_j - x_j - (w_j) + A_j + e_j$, (2) where $e_j = f_{ij} - Y_j$ is the within-ovary error term and is assumed normally distributed with mean = 0 and variance = σ^2 . Using Equation (2) and the fact that $m_j = 1$, $f_j = 2$ $f_{b/m}$, we have (3) Thus the variance around the regression line based upon data set (E, w_j) is composed of two variance components: One is σ^2 and the other is σ^2 , the within-ovary variance. The unbiased estimates of σ^2 , and σ^2 are The parameter q is the number of regression coefficients in the model and n is the number of fish sampled. Most if not all fecundity regressions use only two coefficients (Bagenal 1967) and consequently we use $q = 2$ in this article, and subsequent computations should be redone if $q > 2$. For simplicity, we assume that $M_j = M_j = M$ for $j \neq i$, and of, $\sigma^2 = \sigma^2$, = of. Thus The variance of the sample variance (s^2) is used in the next section for computing the optimum number of ovarian tissue samples for fecundity estimation. B. Optimum number of tissue samples—Since the goodness of fit of any model is measured by s^2 . we chose to minimize the variance of s^2 with respect to the number of tissue samples (m) for a fixed total cost function (Scheffé 1959). $c = c_{li} + c_{lm}$ (7) where c is the total funds available, c_l is the cost of processing a fish, and c_m is the cost of processing a tissue sample Under the assumption of normality of the error terms, one obtains the variance of s^2 as: The optimum sample sizes for selected parameter values are listed in Table 2. The optimum tissue sample size (m) depends on the values of each of the five parameters, $i, t, q, 6 = \sim \$0$, c, c_m , and c^2 . TPble 2.4ptimum number of ovarian tissue samples required for batch fecundity estimation of northern anchovy with two Regression coefficients ($q=2$) and maximum tissue samples > 30 within an m r y ($M > 30$) for w i o u s degrees of data variability ($@ = \sigma^2$, f h) and cost constraints (d e , and c/c_m). Relative index of pressing costs (costs of fish/cost of sample (c/c_m)) * Ratio of Total funds within-ovary available for variance to estimated cost variance per tissue about the line sample $e = 0$ S $e = i$ $e = 2$ $e = 3$ $e = 4$ $e = s$

50	200	lo.m	50	200	10,m	50	200	lo.m	50	200	lo.m	50	200	lo.m	50	200	lo.m	2	2	2	2	2	2	3	3	3	3	4	4	4	5	5	5
6	7	2	2	3	3	3	3	3	3	4	4	4	4	5	4	4	5	6	6	7	5	4											