A key issue in the estimation of fecundity of oviparous fishes is whether or not the annual fecundity can be estimated from the stand- ing 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 synchronouslyprior 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 spawn- ed in the season are usually identifiable because a distinct hiatus in oocyte maturity classes exists between the small, immature, unyolked oocytes that occur the year around and the synchronous- ly maturing annual batch (Hickling and Rutenberg 1936; Yamamoto 1956). Although some of these fishes may spawn repeatedly dur- ing the season, for example, whiting and haddock, the standing stock of yolked eggs is considered representative of the annual fecundity (Hislop 1975; Hislop et al. 1978; Hislop, pers. commun.l). An ex- ception to this occurs when unfavorable conditions result in resorp- tion 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 multi- ple, 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 yolked 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 con- tinuously from small unyolked oocytes to yolked oocytes 0.4–0.7 nun diam., and no large hiatus exists between matur- ity classes of oocytes except for one between hydrated oocytes and advanced yolked 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 b o n g 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 fecund- ity measurement is the number of eggs produced in a single spawn- ing batch (batch fecundity); annual fecundity is a function of both the batch fecundity and the number of spawnings per year. Spawn- ings are so numerous in these fishes that small unyolked 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 an- nual 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 indeter- minate 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 l a b . , P.0 Box 101, Victoria Road, Aberdeen AB9 8DB. Scotland, pen. commun. Oct. 25, 1983 67for fecundity estimation of fishes with seasonally determinate fecund- ity (see, for example, Holden

and Kaitt 1Y74). 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). Vite!logenesis 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 in– crease in dry weight is negligible (LeClus l979a). In northern an– chovy, hydration begins about 12 h before spawning when the eggs are between 0.6 and 0.8 nun (major egg axis) and causes a four–fold increase in wet weight of the ovary as the egg increases to 1.3 nun (major axis) (Hunter and Macewicz 1980; Hunter and Leong 1981) (Fig. 2). Ovulation and spawning soon follow completion of hydra– tion 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. IM 10014 HERRING YINIE S P A I K D EGG SIZE (mm) Figure 1.–Frequency distribution of oocyte dinmeter in the ovaries of herring, pikhnrd (Hidding nnd Rutenberg1936). and northern anchovy (Hunternnd h n g 1981). Herring spawnsn single bntch each year; other species nre multiple–batch spnwners. In the nnchovy, the solid line shows n recently spawned female, the broken linen femalenbout to spawn (just before hydration ofthe oocytes).(From Blnxter nnd Hunter l982.) 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 (day81 Figure Z.–Mnturntion cycle of n U.5 g northern anchovy femnle during peak spawning months where the nvernge interval between spawningsis 7 d. The change in mean wet weight of the o w y b indicated on the left nxis and the mean diameter of the oocytes in the most advanced spawning batch on the right ab. Data from Hunter and Goldberg (1980)and Hunter and Msrmia (1980). 68LABORATORY 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 ad– jacent 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 iden- tifying 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 re- leased 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 estima- tion if the eggs constituting the batch are identified using the egg size-frequency method; however, the volumetric technique is inap- propriate if the hydrated oocyte method is used because Gilson's fluid destroys hydrated eggs. Substantial shrinkage of oocytes oc- curs 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. Al- though 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 in- dividual 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 examina- tion 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 dissec- tion microscope with a lox 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 ex-pressed 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 spawn- ing 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 GI0 mg will in- sure that about I 0 0 oocytes are included in the most advanced modal group of oocytes. All oocytes 20.3 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 5 0 X magnification and measure the oocytes with a rule on the viewing screen of the comparator A starting oocyte size G0.3 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 he included in the count of hydrated oocytes for the hatch fecundity estimate). These hydrated oocytes were 1.2 mm long in the major axis. 70sufficient 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 sec- tion can be used as a guide. ACCURACY AND PRECISION In this section we develop methods to evaluate the accuracy and preci- sion of estimating batch fecundity using the hydrated egg methodol- ogy, 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 selec- tion of an appropriate regression model to express the relation be- tween 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 in- dividual tissue sample also affects precision, but we have not con- sidered this element. We have instead kept the tissue samples within a weight range that yields about 100-200hydrated 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 devia- tion. 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 Table I.-Effect of location of ovarian tissue samples from northern anchovy on the number of hydrated eggs per unit sample weight 0.Effects evaluated by laking tissue samples (n = sample size) from three positions (two ends, I and 111, and middle, 11) from both the right and left ovary. Analysis of variance indicates insignificance of either side or position within a side (SS = sum of squares; MS = mean square). Positions of sample in ovary ,_____ Mean no of eggs/g of ovary tissue Right ovary Left ovary Both ovaries x s n x s n x s n I 5,237 740 12 5,143 1,158 12 5,190 950 24 I1 4,919 790 12 5,243 831 12 5,081 810 24 Ill 5,296 1.005 12 5,540 2,019 12 5,418 1,564 24 A I I 5.151 845 36 5,309 1,396 36 Two-way analysis of variance of eggsig of ovary tissue. SS due tc df SS MS F Right vs. left ovary I 0.0053 0.0053 0.14 Position within ovary 2 0 0268 0.0134 0.34 Interaction 2 0.0269 0.0134 0.34 Error 66 2.5854 0.0392 - Total 71 2.6644 - - - - - -- --~_-.____ ~ 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 com- plete. 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. Varia- tion 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 thc sample variance (or mean square error) around the regression of batch fecundity on female weight (0;) (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, = / (w) + A (1) where the error term (A) has a mean = 0, and variance = 01. Y and Y,, 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 (fi calculated from m ovarian tissue samples per fish. 71Let's denote for the ith fish, i = I , . . . ,n: w, = gonad-free fish weight = fish weight minus gonad weight Y, = total number of hydrated eggs in the ovary y,J = hydrated egg count in the jth tissue sample j = I , . . . ,m z , ~ = weight of jth tissue sample Z , = formalin wet weight of gonad m = number of tissue samples from an ovary M, = maximum number of tissue samples in an ovary f,, = estimated total

number hydrated eggs in the ovary from the f , = estimated total number of hydrated eggs in the ovary = 1 t J / m jth tissue sample = (ytJ/z,,)Z, m j = l , ,?, = sample mean number of hydrated eggs = 1 f i / n i 1 = estimate of batch fecundity from the regression model. Suppose tJis an unbiased estimate of Y,, then t j – Y,+ t J –x – / (w ,) + A , + e , , (2) where e,. = f,, – Y, is the within–ovary error term and is assumed normally distributed with mean = 0 and variance = 0 3 . Using Equation (2) and the fact that m j = l f , = 2 f b / m , we have (3) Thus the variance around the regression line based upon data set (E, w,) is composed of two variance components: One is .'A and the other is o:, the within–ovary variance. The unbiased estimates of 02, and 02 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 regres– sions 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, = M, = M for j # i , and of, = 02, = of. Thus The variance of the sample variance (s:) is used in the next sec– tion 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 i . we chose to minimize the variance of s i with respect to the number of tissue samples (m) for a fixed total cost function (Scheffi 1959). c = c l i i + clmn (7) where c is the total funds available, cI is the cost of processing a fish, and c, is the cost of processing a tissue sample Under the assumption of normality of the error terms, one obtains the variance of $31 as: The optimum sample sizes for selected parameter values are listed in Table 2. The optimum tissue sample size (m) depends on the values of each of the five parameters, i t . , q , 6 = ~$o:, c, c,, and c2. TPble 2.4ptimum number of ovarian tissue samples required for batch fecundity estimntiua of northern anchovy with two Rgmsion coefficients (q=2) and maximum tissue samples >Jo within an m r y (M>30) for w i o u s degrees of data variability (@=o,f hi)and cost constraints (d e , and c,/c,). Relative index of pnressing costs (costs of fishkost of sample (c,Ic2)* Ratio of Total funds within–ovary available for variance to estimated cost variance per tissue about the line sample e = O 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 10,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 5 4 4 5 6 6 7 5 4