Procedures to Estimate Fecundity of Marine Fish Species in Relation to their Reproductive Strategy

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Abstract

Appraisal of reproductive strategy and fecundity is necessary to evaluate the reproductive potential of individual fish species. To estimate reproductive potential, one needs to consider a variety of attributes including onset of maturity, fecundity, atresia, duration of reproductive season, daily spawning behaviour and spawning fraction. In this contribution, we review several methods currently used to estimate fecundity of marine fishes collected in the field in relation to their reproductive strategy. The advantages and disadvantages of each method are provided. Requirements are given to appropriately sample gonadal tissue that will enable researchers to establish incidence of sexual maturity and estimate fecundity.

Key words: atresia, fecundity estimation, marine fish, maturity ogive, North Atlantic, oocyte histology, reproductive potential, reproductive strategy, sampling, spawning fraction

Introduction

Descriptions of reproductive strategies and the assessment of fecundity are fundamental topics in the study of the biology and population dynamics of fish species (Hunter *et al.*, 1992). Studies on reproduction, including the assessment of size at maturity, fecundity, duration of reproductive season, daily spawning behaviour and spawning fraction, permit quantification of the reproductive capacity of individual fish. This information in combination with estimates of egg production at sea enable estimation of spawning stock biomass (Saville, 1964; Parker, 1980; Lasker, 1985). This increases our knowledge about the state of a stock and improves standard assessments of many commercially valuable fish species. Moreover, establishment of extensive data bases on reproductive parameters with corresponding data on abiotic factors enables the study of causal relationships between reproductive potential and environmental variation. This leads to a better understanding of observed fluctuations in reproductive output and enhances our ability to estimate recruitment (Kraus *et al.*, 2002).

Marked differences in fecundity among species often reflect different reproductive strategies (Pitcher and Hart, 1982; Wootton, 1984; Helfman *et al.*, 1997; Murua and Saborido-Rey, 2003). Within a given species, fecundity may vary as a result of different adaptations to environmental habitats (Witthames *et*

al., 1995). Even within a stock, fecundity is known to vary annually, undergo long-term changes (Horwood *et al.*, 1986; Rijnsdorp 1991; Kjesbu *et al.*, 1998) and has been shown to be proportional to fish size (and hence, age) and condition. Larger fish produce more eggs, both in absolute and in relative terms to body mass. For a given size, females in better condition exhibit higher fecundity (Kjesbu *et al.*, 1991). Fish size and condition are, thus, key parameters to properly assess fecundity at the population level. In heavily exploited populations, large old fish will be eliminated more rapidly because they are exposed to size-selective fishing mortality (Trippel, 1999). In this situation, population fecundity not only declines as a consequence of the reduced abundance of spawners, but also due to the disproportionate reduction in large, highly fecund females. Values of condition indices vary among individuals, and may vary annually within individuals. Changes in environmental factors, such as temperature, may affect condition by influencing fish behaviour and metabolism, as well as food availability. Declines in fecundity due to reduced condition can be reflected in a lower number of oocytes that develop in a given breeding season or through atresia. In extreme cases, low condition can induce reproductive failure and lead to skipped spawning seasons (Bell *et al.*, 1992; Livingston *et al.*, 1997). Fecundity and atresia can also be affected by environmental pollution (Johnson *et al.*, 1998). In light of these issues, regular estimation of fecundity, in conjunction with environmental and other biological data, would not only help to understand the underlying mechanisms regulating annual variability in fecundity, but could possibly help to explain variability in recruitment.

The aim of this review is to provide a protocol to estimate the annual fecundity (see Table 1 for definitions) of commercially important marine fish species from field samples. The protocol is divided into two sections. The first deals with fish sampling and preservation of gonads. The second part is focused on methods to estimate annual fecundity in relation to reproductive strategy as described in Murua and Saborido-Rey (2003). The suitability of different methods specific to each reproductive strategy are discussed and recommendations are made.

Sampling

Sampling of fish from a population for maturity and fecundity studies should provide an accurate representation of the seasonal and regional distribution of individuals in relation to spawning activity for all areas considered in the study. Sampling should span the entire range in body length, the total distribution area and account for variations in the timing of maturation and spawning of different stock components (e.g., sub-stocks and age classes).

Species, and even different populations within a species, show differences in size at maturity, growth rate, and spawning time. Hence, it is beyond the scope of the present contribution to provide reasonable guidelines for each stock. Investigators interested in pursuing this should scan the relevant assembled literature specific to a stock before designing a specific sampling strategy (for example, Tomkiewicz *et al.* (2003a): data available on 42 fish stocks of the Northwest Atlantic).

Although the main purpose of this contribution is to provide guidelines to estimate fecundity of marine species exhibiting different reproductive strategies, data on sex ratio and sexual maturity are also fundamental to the estimation of population fecundity. A brief description of requirements for sampling to assess sexual maturity is thus presented followed by sections on procedures for fecundity sampling.

Sampling for maturity ogive

The maturity ogive is based on a classification of the population into mature (fish that certainly will or have spawned) and immature individuals in relation to age or length. It has been reported in the case of sole (*Solea solea*) (Ramsay and Witthames, 1996), that some females are partially mature by commencing vitellogenesis amongst the leading oocyte cohort but later aborting the development without producing batches of eggs. It has been hypothesized that this phenomenon may occur in other teleosts, being most abundant among individuals in the year-class maturing for the first time. These females have been defined as mature inactive (Hunter *et al.*, 1992). The fact that some mature females fail to spawn in a given breeding season should be considered when applying maturity ogives to estimate spawning stock biomass.

During the spawning period, samples from the spawning aggregations may primarily consist of adults because of different spatial separation or catchabilities of mature and immature individuals. Consequently, sampling the spawning aggregation may result in under representation of immature fish of maturing cohorts; and therefore the estimated maturity ogive (e.g., size or age at 50% maturity) will be biased low.

TABLE 1. Definitions related to reproductive biology and fecundity terms used within this review (for further details see Murua and Saborido-Rey, 2003).

Fecundity terms	Definition	Reference
Total spawner species	The whole clutch of developed oocytes is shed in an unique event or over a short period of time but as part of a single episode.	Holden and Raitt (1974)
Batch spawner species	The eggs are released in batches usually over a protracted spawning period (weeks or months). Only a portion of the yolked oocytes is selected to be spawned and hydrated in each batch.	Holden and Raitt (1974)
Synchronous ovary	All oocytes, once formed, grow and ovulate from the ovary development in unison; further replenishment of one stage by an earlier stage does not take place.	Marza (1938); Wallace and Selman (1981)
Group-synchronous development	At least two cohorts of oocytes can be distinguished in the maturing ovary; a fairly synchronous population of larger oocytes (defined as a "clutch") and a more heterogeneous population of smaller oocytes from which the clutch is recruited. The former are the oocytes to be spawned during the current breeding season, while the latter are the oocytes to be spawned in future breeding seasons.	Marza (1938); Wallace and Selman (1981);
Asynchronous ovary development	Oocytes of all stages are present in the ovary without dominant populations. The ovary appears to be a random mixture of oocytes, at every conceivable stage.	Marza (1938); Wallace and Selman (1981)
Determinate fecundity	In fishes with determinate fecundity, the standing stock of yolked oocytes (total fecundity) prior to the onset of spawning is considered to be equivalent to the potential annual fecundity.	Hunter *et al.* (1992)
Indeterminate fecundity	This term refers to species where potential annual fecundity is not fixed before the onset of spawning and unyolked oocytes continue to be matured and spawned during the spawning season	Hunter *et al.,* (1992)
De novo vitellogenesis	The process of producing vitellogenenic oocytes from previtellogenic oocytes during the spawning season, and consequent recruitment into the standing stock of yolked oocytes	Hunter and Goldberg (1980)
Annual fecundity or annual realized fecundity	The total number of eggs released per female in a year.	Hunter *et al.* (1992)
Annual population fecundity	The total number of eggs produced by a population in a breeding season	Bagenal (1978)
Potential annual fecundity	The total number of advanced yolked oocytes matured per female and year, uncorrected for atretic losses	Macer (1974); Hunter *et al.* (1992)
Total fecundity	The total number of vitellogenic or advanced yolked oocytes at any time in the ovary.	Hunter *et al.* (1992)
Residual fecundity or remnant fecundity	The number of vitellogenic or advanced yolked oocytes in ovaries showing postovulatory follicles. This indicates that these females had already spawned some eggs.	Murua and Motos (1998)
Batch fecundity	The number of eggs spawned per batch. The sum of batch fecundities represents the realized annual fecundity.	De Vlaming (1983)
Spawning fraction	Fraction of mature females spawning per day	Alheit (1985)
Postovulatory follicle	After ovulation, the follicle tissue that encapsulated each hydrated oocyte collapses, and remains in the ovary as an evacuated follicle. These structures are used as indicators of previous spawning activity.	Hunter and Macewicz (1985)

TABLE 1.   (Continued). Definitions related to reproductive biology and fecundity terms used within this review (for further details see Murua and Saborido-Rey, 2003).

Fecundity terms	Definition	Reference
Atresia	The process of oocyte and follicle resorption altering the oocyte structure as an indicator for the destruction and resorption of oocytes.	Bagenal, (1978)
Alpha atresia	The initial phase of oocyte atresia. In this phase, the oocyte is resorbed, leaving only the follicular layers.	Hunter and Macewicz (1985)
Turnover rates of atretic oocytes	Duration of specific atretic stages of oocyte resorption.	Hunter *et al.* (1985); Kjesbu *et al.* (1991)

In order to obtain representative ogives, sampling should be conducted over the entire stock's spatial distribution, which may be characterized according to age and length (ICES, MS 1997). Additionally, the maturity ogive, determined after sampling of spawning and juvenile areas, should be weighted according to differences in population density (Armstrong *et al.*, 2001).

Computation of accurate maturity ogives requires accurate maturity staging (Table 2). There is considerable concern about the reliability of macroscopic gonadal grading, and hence it is desirable to increase the precision and confidence in the assessment of reproductive status and resulting maturity ogives. In this regard the separation of fish classified as immature or partially mature from those adults staged as post-spawning or resting, and the determination at which time of the maturation cycle such discrimination can be made (i.e., in prespawning, peak spawning, etc.) is of particular importance. The use of histological techniques to study gonadal maturation and to estimate the length or age at maturity has proven to have greater precision than traditional macroscopic techniques and is recommended as a possible substitute or augmentation to visual classification (Murua and Motos, 1998; Saborido-Rey and Junquera, 1998; Tomkiewicz *et al.*, 2003b).

When establishing a maturity sampling scheme, it is crucial to include those length and/or age groups within the transition from immature to mature. The length or age transition range from immature to mature, where the slope of the maturity curve is steepest, requires fine resolution and collection of sufficiently large sample sizes to estimate reliable proportions of mature individuals.

Thus, regarding maturity assessment, we strongly recommend sampling the entire distributional area of a given stock (including adult and juvenile areas) and the use of histological criteria for the classification of gonads to replace (or at least verify) a less costly more extensive survey based on macroscopic criteria (Table 2). Samples should be taken during pre-spawning, preferably when the population is about to spawn, and during the spawning season but before peak spawning time to evaluate mature inactive fish. Accurate assessment of maturity outside the spawning period could be conducted during the vitellogenesis period long before spawning begins (see Murua and Saborido-Rey, 2003) or even during the resting period. But the latter case is only applicable after extensive evaluation of the parameters used (Saborido-Rey and Junquera, 1998; Murua and Motos, 2000). The latter authors and others (Woodhead and Woodhead, 1965; Zamarro *et al.*, MS 1993) reported that postovulatory follicles can be identified in post-spawning fish up to 3 months or longer after the spawning season has finished in wild populations, but laboratory studies are also needed to evaluate this methodology.

When first undertaking maturity assessment of a stock, it may be necessary to collect a large sample size of each age group. After this intensive study, it may be possible to further refine sampling strategy by age, season and area in order to reduce the overall effort, without a reduction in accuracy.

Sampling for fecundity

Estimation of fecundity involves a number of underlying assumptions, and these may differ depending on the species' reproductive strategy (Hunter *et al.*, 1992; Murua and Saborido-Rey, 2003). Fish reproduction is also highly adaptive with locality

TABLE 2. General macroscopic maturity classification criteria of fish ovaries and corresponding histological descriptions (for histological definitions see Appendix 2). To avoid misclassification, due to the difficulty to classify ovaries correctly based on gross anatomical examination, a simple six scale macroscopic classification is presented. Macroscopic staging is most accurate when stages 3–5 predominate within a population which is just before or during the first part of a spawning season.

Females:

Maturity stages	Probability of correct classification into mature or immature based on macroscopic criteria	Macroscopic Characteristics	Microscopic Characteristics
Immature 1	High	Small ovaries without visible oocytes. Ovarian wall thin. Separating this stage from the stage of onset of maturity is often difficult.	All the oocytes in the previtellogenic stage (primary growth stage)
Maturing 2	Low Confused with stages 5 and 6	Medium size ovaries occupying 1/4 to 3/4 body cavity. Visible opaque oocytes (0.2 mm to 0.5 mm resolution of human eye).	The maturation process has started and the most advanced oocytes are in cortical alveoli stage or early vitellogenic stages.
Mature 3	High	Large ovaries occupying 3/4 to almost filling body cavity with blood capillaries. Yellow/orange colored. Visible opaque oocytes (0.5 mm resolution of human eye), without bruised areas.	The most advanced mode of oocytes within the ovary is in vitellogenesis stages.
Spawning 4	High	Translucent oocytes that may flow or not on applying pressure. Hydrated oocytes are larger than the opaque oocytes.	Spawning is imminent and the oocytes are either in migratory nucleus stage or hydration stage.
Resting 5	Low Confused with stages 2 and 6	Bruised ovary. Purple in color and very flaccid. Occasionally with remaining translucent oocytes. But still the advanced oocytes for the next batches are visible opaque oocytes (0.5 mm resolution of human eye, as big yellow oocytes).	Some eggs have been released and post-ovulatory follicles are present. Some remaining hydrated oocytes, from the previous batch, may appear. Further batches of hydrated oocytes will be produced.
Spent 6	Low Confused with stages 5 and 2	Bruised ovary. Purple in color and very flaccid. Ovary wall thick and blood capillaries are big. There are no more advanced oocytes remaining in the ovary.	The last batch has been released. Oogonia and chromatin nuclear stages oocytes are present or if more advanced oocytes are present they will undergo generalized atresia and will be resorbed.

(Witthames and Greer Walker, 1995) and before commencing any routine study a more detailed investigation of the dynamics of oocyte growth, maturation and egg production should be undertaken for the population in question. It is therefore necessary to adjust the fecundity estimation approach and the sampling program according to reproductive strategy to make the most informed choice of method. We emphasize some important requirements regarding fecundity sampling for the two main reproductive strategies: determinate and indeterminate fecundity.

For species with determinate fecundity a basic assumption is that the fecundity is fixed before the

onset of spawning, so that the potential fecundity is equivalent to the annual egg production after accounting for atretic losses (Hunter *et al.*, 1992). Consequently, atresia needs to be discounted from potential fecundity to determine realized annual fecundity.

For individuals exhibiting determinate fecundity, the oocytes that constitute the potential annual fecundity have to be identified with certainty. Time of sampling in relation to the annual maturation cycle is important in this regard. When an ovary is sampled too early in the season, it may not be sufficiently developed to allow the identification of all oocytes destined to be spawned, i.e., the gap in the oocyte size frequency distribution may not be clear and a bias may be introduced because not all oocytes have been recruited into the annual stock of advanced oocytes, or, on the contrary, some oocytes that will not develop further become included in the counts. When sampling during the spawning season for those species that exhibit determinate fecundity and batch spawning, undetected spawned batches may result in underestimated fecundity. To exclude both possible sources of bias, the timing of sampling has to be adjusted so that only ovaries in the optimal developmental stage are considered.

These optimal stages for determinate spawners are pre-spawning individuals in late vitellogenesis (stage 3, Table 2) (May, 1967; Hunter *et al.*, 1992; Kraus *et al.*, 2000). Inherent with this approach is that one should account for body size-specific differences in spawning schedules within a stock. Although for most stocks a spawning peak can be identified, the entire spawning population will also contain early and late spawning sub-components (e.g., based on genetics, body size, etc.). A possible bias in fecundity estimates may arise, when these sub-components vary in their size-specific fecundity and are not sampled representatively. A sampling campaign should, thus, either cover the entire pre-spawning and spawning period or should at least be scheduled to meet the spawning peak so that fecundity is representatively assessed for the entire stock. Because fecundity has to be estimated from pre-spawning ovaries, histological screening to assess if a female has already started spawning should be performed. The presence of postovulatory follicles (POF) or hydrated oocytes in the ovary (see Appendix 2 for further details on histology) indicate that the spawning process has already begun. To apply these criteria it is important to ensure that the POF persist at least as long as the time interval between batches.

One of the main goals of estimating fecundity, independent of reproductive strategy, is to establish a fecundity-size (length/weight/age) relationship, which can be used to scale estimates of spawning stock biomass or spawner abundance to population egg production. For this purpose, it is very important to ensure that the entire length range of females present in the population is covered providing consistent sampling among subcomponents over years. This is best achieved through length-stratified sampling over the stock's entire size distribution.

The term indeterminate fecundity refers to species in which the potential annual fecundity is not fixed before the onset of spawning (Hunter *et al.*, 1992). In such species, previtellogenic oocytes may develop and recruit into the standing stock of yolked oocytes at any time during the spawning season (vitellogenesis *de novo*; Hunter and Goldberg, 1980). Therefore, the estimation of potential fecundity in the ovary prior to the onset of spawning is meaningless. In these species, annual fecundity should be estimated from the number of oocytes released per spawning (batch fecundity), the percentage of females spawning per day (spawning frequency), and the duration of the spawning season (Hunter *et al.*, 1985).

Clearly, for species characterized by indeterminate fecundity, a different sampling strategy has to be applied in order to obtain samples to estimate batch fecundity, spawning fraction and sex ratio. Again, a length-stratified sampling scheme is recommended to estimate batch fecundity, as it would cover the entire size distribution of a stock. The sampling for batch fecundity analysis would also assure hydrated females in adequate numbers to cover the entire size range of a stock. For estimating the spawning fraction and sex ratio, mature females should be selected at random. In the latter approach, fishing gear and timing of sampling should be designed in order to provide a representative sample of the reproductive population. An examination of fish movements (e.g., using tagging) may be required to investigate whether individuals remain in the survey area during the period of investigation. Mackerel (*Scomber scombrus*) in the eastern Atlantic, for example, include members of the same population that spawn south of 40° N in January and north of 60° N in July. Dawson (1986) showed a succession of mackerel females by size in the central

spawning area and proposed an annual migration cycle in which adults moved into and out of the spawning area.

The number of individuals per size class necessary to obtain reliable size-specific fecundity estimates is dependent on the fecundity modality, i.e., for indeterminate species, a high number of samples per size class is required to account for the relatively large seasonal variation in batch fecundity (i.e., sampling should be repeated on a regular basis over the spawning season). However, sample sizes are highly dependent on the degree of individual variation in fecundity, which could differ strongly between stocks or species, and within a given stock among sizes. Thus, no general rule is recommended for sample sizes, but sampling should be adequately planned to ensure significance of fecundity-size relationships.

Reliable estimates of the spawning fraction not only depend on the number of individuals sampled at each station, but also on the total number of stations. According to Picquelle (1985), it is generally more efficient to increase the number of trawl stations and reduce the sample sizes per station than *vice-versa*. The appropriate number of fish at each station and the necessary number of stations to compute the spawning frequency with sufficient precision are discussed in detail in Picquelle (1985).

In summary, sampling gonads for fecundity analysis has to be adjusted according to the reproductive strategy of the species under investigation, and even within a specific reproductive strategy it will depend on the parameter to be estimated. The sampling protocol provided in Appendix 1 covers the different reproductive strategies. However, fine adjustments may be required for each stock under investigation.

Fecundity Estimation

In this section we review methods for estimating fecundity (total potential fecundity, batch fecundity), spawning fraction and atresia from field samples. First, different methods to estimate fecundity are listed including a short description followed by a summary table of the advantages and disadvantages of each method (Table 3). Second, problems in estimating the amount of atresia and spawning fraction are discussed. Finally, combining procedures to estimate fecundity, spawning fraction and atresia, we focus on estimating the realized annual fecundity (see Table 1 for definitions).

Estimation Methods

According to the species under investigation and available laboratory facilities, investigators should judge which of the methods described below is applicable. Depending on the peculiarities of a given species small changes in the methodology may be required. For example, it is possible to use the gravimetric method to estimate the potential fecundity for any species, but the threshold diameter at which the developing oocytes could be separated from pre-vitellogenic oocytes will be variable. Consequently, prior knowledge of the reproductive biology of the species is essential.

Prior to the application of any fecundity estimation method, homogeneity in oocyte distribution in the ovary should be investigated to ensure that the sub-sample to be analysed represents the entire ovary, e.g., test for differences in oocyte density within an ovary lobule and between ovary lobules. For example, it has been shown that oocyte size, and hence fecundity per unit volume, in yellowfin sole (*Limanda aspera*) (Nichol and Acuna, 2001) and plaice (*Pleuronectes platessa*) (Witthames, unpubl. data) vary along the axis of each ovarian lobe.

Gravimetric method. The gravimetric method is currently the most common method used to estimate fecundity. It is based on the relation between ovary weight and the oocyte density in the ovary. This method can be used to estimate batch fecundity, total fecundity and potential annual fecundity. For more details see Hunter and Goldberg (1980) and Hunter *et al.* (1989).

Using this method, fecundity (*F*) is determined as the product of gonad weight and oocyte density. Oocyte density is the number of oocytes per gram of ovarian tissue, and it is determined by counting the number of oocytes (o_i) in a weighed sample of ovarian tissue. After weighing the ovaries (W_{ovary}), 3–5 subsamples of known weight are extracted from different parts of the ovary lobe. The accuracy and precision of fecundity estimation should be evaluated, especially regarding the number of sub-samples (for further details see Hunter *et al.*, 1985). As a rule-of-thumb, a sufficient number of subsamples is reached when the CV of the n^o of oocytes per unit weight is less than 5% (Kjesbu, 1989). Each subsample is weighed (w_i) to the nearest 0.001 g and then dispersed with a fine paint brush, or light air pressure created by repeatedly sucking in and out of a Pasteur pipette, to identify and count all the vitellogenic oocytes.

Oocytes can be counted (and at the same time measured) using a stereoscopic microscope with a grid or using any image analysis system software.

$$F = \frac{\left[\sum_i \frac{o_i}{w_i}\right]}{n} * W_{ovary}$$

Variations of this method are related to whether total fecundity, batch fecundity or potential annual fecundity is estimated and the type of oocytes enumerated. To estimate batch fecundity the hydrated oocytes within the subsamples are counted, while for calculations of total fecundity or potential annual fecundity the advanced yolked oocytes (including the hydrated oocytes) are counted. As we stated above, in the case of species where no clear gap between previtellogenic and vitellogenic oocytes is present (Fig. 1 and 2), e.g., hake (*Merlucius merlucius*) or mackerel, only oocytes larger than a critical minimun diameter are included for potential or total fecundity estimations. This diameter marks the transition from previtellogenic to vitellogenic oocyte (Khoo, 1979) from where the oocytes comprising the fecundity become steadily larger and more opaque as yolk and cortical alveoli accumulate.

To estimate potential or batch fecundity, ovaries should be screened histologically to check for the occurrence of post-ovulatory follicles (POF) (Fig. 3). Ovaries containing POFs should be eliminated from potential fecundity calculations, since the presence of POFs indicate that spawning has already started and the number of oocytes in the ovary has consequently decreased. For batch fecundity estimations, only hydrated ovaries which do not contain early stage POFs (Hunter and Macewicz, 1985) should be used because the presence of these follicles indicate that some eggs have been already released.

Volumetric method. The volumetric method is based on the same principles as the gravimetric method, but uses ovarian volume and the subsample volume instead of ovary weight and subsample weight (Simpson, 1951).

Combined gravimetric and automated particle counting method. This method is a variation of the gravimetric method; the major difference being that an automated particle counter is used to enumerate the number of oocytes in a subsample (Witthames and Greer Walker, 1987; Kraus *et al.,* 2000).
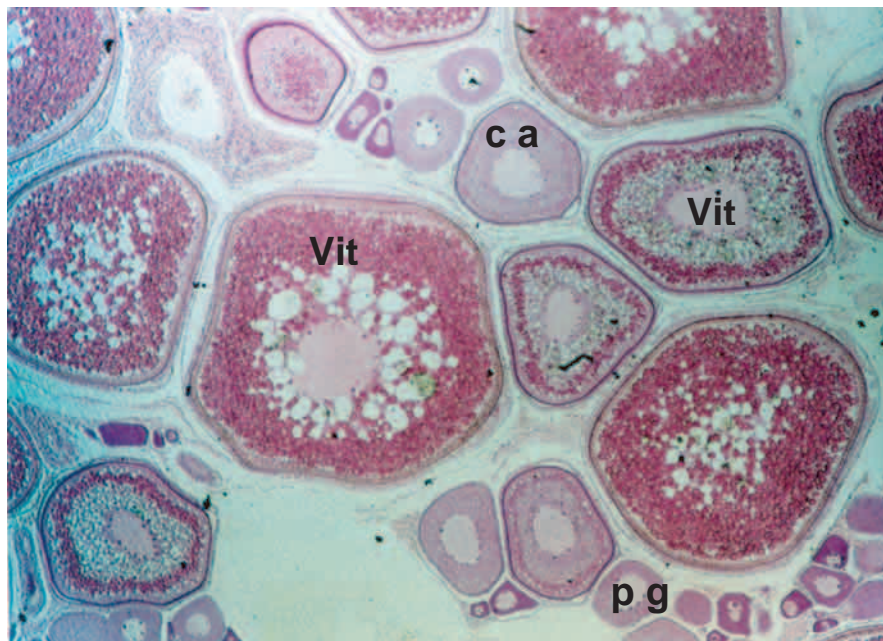

Fig. 1. Histological sections of ovaries showing oocytes at various stages of development: previtellogenic primary growth oocytes (pg), cortical alveoli oocytes (ca), and vitellogenic oocytes (Vit.) (for further details see Fig. 1 in Murua and Saborido-Rey, 2003).
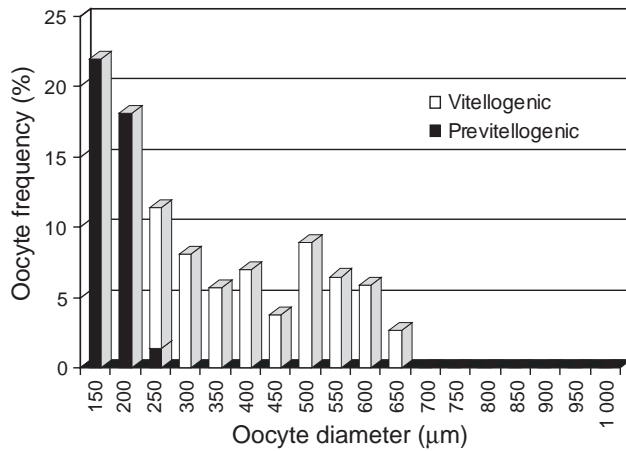
Fig. 2. Ooctye size frequency distribution of previtellogenic oocytes and vitellogenic oocytes of mature hake, *Merluccius merluccius*, female in stage 3 of maturation (Source: Murua and Motos, 1998).

The advantage of using an automatic particle counter to determine the number of oocytes in a subsample is that the counting procedure is less time consuming. Hence, the sample sizes are commonly much larger or the whole ovary could be analyzed compared to manual counts. This enables greater precision in weight measurements of the comparatively large counted oocyte fraction. However, the precision of automated particle counters must be evaluated according to the prevailing operating conditions (count rate, debris or bubbles in the carrier fluid) and compared to manual counts. For example, a Hiac optical particle sensor and pulse height analyzer have been shown to count samples with a CV of 0.6% (Witthames and Greer Walker, 1987). A similar precision was obtained using a Macro-Flow-Planctometer (Hüller *et al.,* 1991) with a CV of 0.4% for repeated counts and an average deviation of

automated from stereomicroscope counts of 1.4% (Kraus, 1997).

Stereometric method. From histological sections it is possible to determine the number of cells in different categories, i.e., oocytes that are either previtellogenic, vitellogenic or atretic; by applying the principles of stereology (Emerson *et al.*, 1991). The stereological method of Emerson *et al.* (1991) is based on the Delesse principle (Delesse 1847) which states that the fractional volume (V_i) of a component (i) is proportional to its fractional cross sectional area (A_i). An underlying assumption of this principle is that the component is distributed randomly and evenly through the tissue.

The abundance of a specific type of oocyte, defined by morphological criteria, per unit volume is measured using the stereological method developed by Weibel and Gomez (1962) (Fig. 4). The following equation describes the relationship between fecundity (F) and its dependent variables (Weibel *et al.*, 1966):

where:

$$F = O_v \times \frac{K}{\beta} \times \frac{N_a^{3/2}}{V_i^{1/2}}$$

β is a shape coefficient, i.e., ratio between the longest and shortest axis of the oocytes transected

O_v is the ovary volume,

N_a is the number of oocyte (previtellogenic, vitellogenic, atretic) transections per unit area,

V_i is the partial area of oocytes (previtellogenic, vitellogenic, atresia) in the histological section,

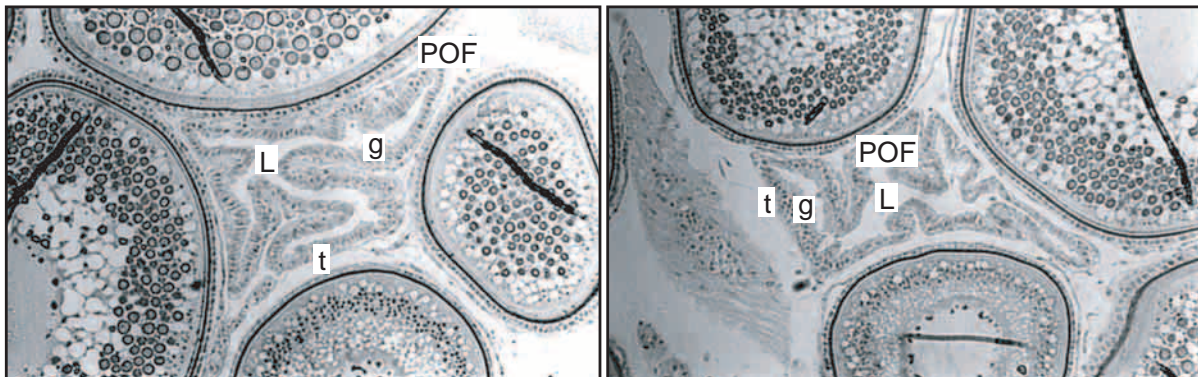K a size distribution coefficient,

Fig. 3. Histological sections of ovaries showing postovulatory follicles (POFs) at various stages of resorption (for further details see Appendix 2).
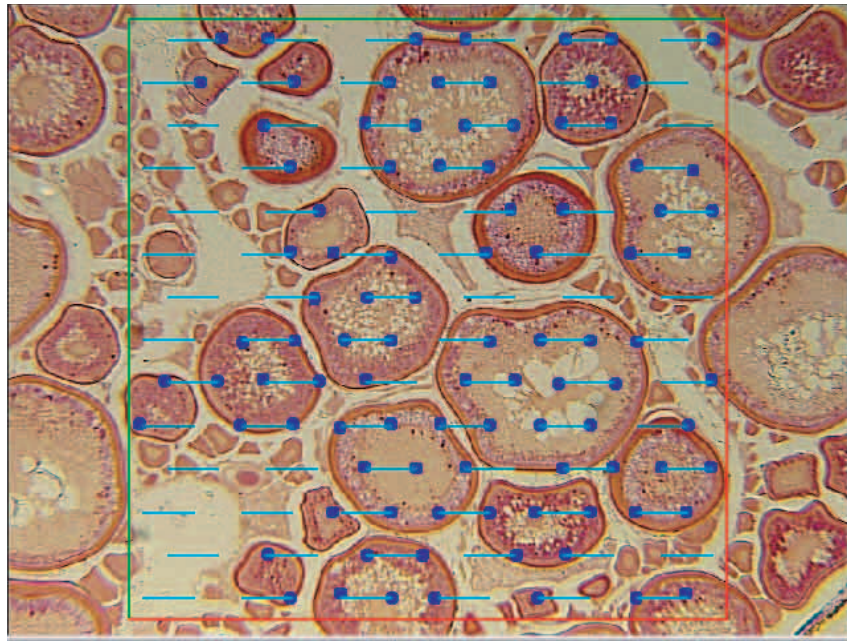
Fig. 4.   Example of a Weibel grid overlaid on the image of section showing point counts to determine the partial volume (V_i) of vitellogenic oocytes in the ovary. In this grid there are 84 bars and the end of each bar represents a test point (n = 168). (V_i = 97/168) The parameter N_a is the number of vitellogenic oocytes transected in the grid area and includes oocytes overlapping the green borders but not the red borders of the grid.

$$K = \left[\frac{M_3}{M_1}\right]^{3/2}$$

M_1 is the mean oocyte diameter

$$M_1 = \left[(D_1 + D_2 + D_n)/n\right]$$

M_3 is the cube root of the third moment about the mean of the oocyte distribution.

$$M_3 = \left[\frac{(D_1)^3 + (D_2^{\ 3}) + (D_n^{\ 3})}{n}\right]^{1/3}$$

The parameter K is determined, using the formula of Williams (1997), by measuring the oocyte diameter, as the mean of the largest and smallest diameters of an oocyte, in a sample of vitellogenic oocytes transected through the nucleus. Emerson et al., (1991) stated that measuring 50 oocytes is sufficient to give a stable value for K of an ovary.

This estimation of oocyte size frequency assumes that the nucleus diameter is constant over the whole size range of vitellogenic oocytes measured (Greer Walker et. al., 1994) (Fig. 2). However, not all species conform to this criterion. Data to correct the observed oocyte diameter in a section to the real diameter are available for mackerel (Greer Walker et al., 1994), bass (Dicentrarchus labrax), cod (Gadus morhua), and sole (Witthames, unpubl. data). Where the data are not available, the relationship between oocyte and nucleus diameter should be determined from histological sections. Estimation of atretic oocytes requires special mention because it is not possible to determine atretic oocyte size frequency and certain assumptions must be made which are discussed in the section headed atresia.

The proportional area (A_i), and hence the proportional volume (V_i), occupied by vitellogenic oocytes is determined by counting the number of points (p_o) on the Weibel grid (which overlays the oocytes of each stage) and dividing this number by the total number of points on the grid (p_t). The number of oocytes per unit area (N_a) is estimated by counting the number of oocytes of each stage within the total area of each grid (Emerson et al., 1991). New developments in image analysis and associated software have reduced the required labour costs

considerably as direct or even automatic area measurements are now possible.

Auto-diametric fecundity method. This method estimates the potential fecundity from the mean vitellogenic oocyte diameter and the total ovary weight using a calibration curve that relates mean oocyte diameter to oocyte density (Thorsen and Kjesbu, 2001). The mean oocyte diameter is measured from whole oocytes of preserved ovaries (buffered formalin) using an image analysis system (Fig. 5). The measurement technique is based on light thresholding of the dark vitellogenic oocytes using background illumination. The method has to be established for each species by generating regression equations between mean pre-spawning oocyte diameter and the number of oocytes per gram ovary in pre-spawning

fish. Once this calibration regression has been established, measuring fecundity with this method is rapid, typically requiring about 5 min for each sample. The method does not require subsample weighing, which eases the collection of samples at sea. Compared to the gravimetric method, the auto-diametric method requires additional electronic equipment and thus is more costly. The establishment of a standardized measuring procedure and a calibration curve may take considerable time. In conclusion, this method would only be a valuable rapid alternative if fecundity can be assessed routinely and is most appropriate for species with group-synchronous ovaries (in these species the gap between developing yolked oocytes and previtellogenic oocytes is very clear, and therefore, the maturing oocytes are clearly separated in size and darkness from the earlier oocyte stages).

TABLE 3. Features, advantages and disadvantages of methods used to estimate fecundity of wild collected fish species.

Method	Advantages	Disadvantages
Gravimetric	• Accurate and inexpensive low technological approach. • Can provide additional information on oocyte frequency and oocyte diameter. • Very useful technique for batch fecundity estimations.	• No information on atresia or about presence of spawning markers such as POFs. • Not good for species with asynchronous development of oocytes, or where the gap between advanced vitellogenic oocytes and previtellogenic oocytes is poorly developed as in less developed ovaries. • Requires the whole ovaries to be returned to the laboratory. • Requires histological analysis to estimate proportion of atretic to vitellogenic oocytes and to identify presence of POFs. • Time consuming.
Volumetric	• Inexpensive low technological approach. • Can provide additional information on oocyte frequency and oocyte diameter.	• No information on atresia or about presence of spawning markers such as POFs. • Not good for species with asynchronous development of oocytes, or where the gap between advanced vitellogenic oocytes and previtellogenic oocytes is poorly developed as in less developed ovaries. • Requires the whole ovary to be returned to the laboratory. • Requires histological analysis to estimate proportion of atretic to vitellogenic oocytes and to identify presence of POFs. • Time consuming.

TABLE 3. (Continued). Features, advantages and disadvantages of methods used to estimate fecundity of wild collected fish species.

| Method	Advantages	Disadvantages
Combined gravimetric/ volumetric and automated particle counting method	• Variation of the gravimetric/volumetric method enables the counting of large amounts of oocytes in a short time, which enhances accuracy. • Can provide additional information on oocyte size frequency.	• Difficult to tease oocytes apart. • Requires Gilson's fixative (highly toxic containing mercuric chloride) or enzymatic disintegration to tease oocytes apart. • No information on atresia or about presence of spawning markers such as POFS. • Requires the whole ovary to be returned to the laboratory. • Requires histological analysis to estimate proportion of atretic to vitellogenic oocytes and to identify presence of POFs. • Expensive electronic equipment necessary and needs calibration.
Stereometric	• Complete analysis includes spawning status, atresia, fecundity, egg size, and numbers of previtellogenic oocytes. • Includes identification of advanced vitellogenic oocytes based on the staining properties and size of oocytes.	• Not good for large ovaries >200 g. • Requires expensive high technological instruments expenditure on image analysis or PC / video equipment. • Requires the whole ovary to be returned to the laboratory. • Not valid for batch fecundity estimations.
Disector	• Analyis for atresia estimation. • Sections provide histological information including the presence and number of post ovulatory follicles	• Very time consuming and requires serial sectioning of ovary fragments.
Auto-diametric fecundity method	• Analysis includes spawning status, fecundity and egg size. • Highly time efficient method to estimate fecundity. • Does not require the whole ovary to be returned to the lab providing savings in space and fixative.	• Methods published but have to be validated for more species and could be only used for species with determinate fecundity to estimate potential fecundity in prespawning fishes, i.e. when the hiatus between previtellogenic and advanced vitellogenic oocytes is developed. • Not validated for species with asynchronous development of oocytes (Murua and Saborido-Rey, 2003). • Not validated for ovaries towards the end of spawning and or where there are high numbers of atretic oocytes or POFs. • Requires histological analysis to estimate proportion of atretic to vitellogenic oocytes and to identify presence of POFs.
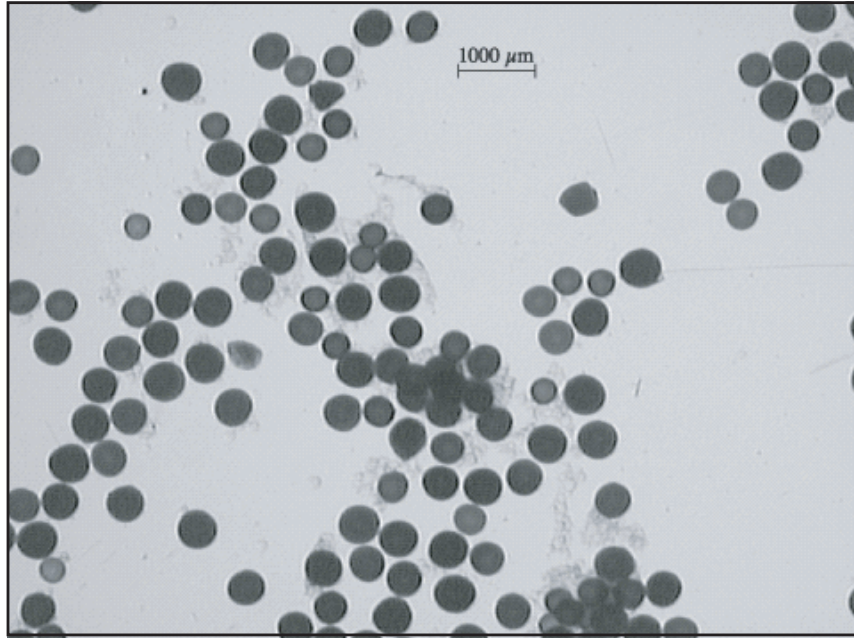
Fig. 5. Whole mount picture (Source: Thorsen and Kjesbu, 2001). The auto-diametric fecundity method is based on automatic measurements of mean vitellogenic oocyte diameter from whole mount photographs. The image analyzer software distinguishes single vitellogenic (dark) oocytes from small previtellogenic (light) oocytes, clusters of vitellogenic oocytes, and bits of connective tissue using thresholds of particle size, darkness and a roundness factor.

Spawning Fraction

In fishes with indeterminate fecundity, the annual fecundity should be estimated from the combination of batch fecundity, spawning fraction and the duration of spawning season. The most accurate way to estimate the spawning frequency (batch interval) is to follow the batch production sequences of an individual fish in tank experiments (Hunter and Goldberg, 1980; Kjesbu *et al.*, 1991). If this approach cannot be applied, the fraction of females spawning per day (spawning fraction) could be assessed from the prevalence of spawning stages determined from a random sample of gonads (Hunter and Goldberg, 1980; Hunter and Macewicz, 1985; Priede and Watson, 1993) as follows:

$$S = 24 * \frac{S_i}{t_i}$$

and hence,

Batch Interval = 1/S

where S_i is the prevalence of the *i*-th ovary stage and t_i is the duration of the *i*-th stage in hours. In principle,

any of the ovary stages associated with spawning can be used, provided its duration is known (Priede and Watson, 1993).

The postovulatory follicles (POFs, Fig. 2) are the most common ovarian features used to estimate the spawning fraction. It is necessary to age the deterioration and resorption processes of the POF with a series of distinct histological stages, where each stage indicates the time elapsed since spawning (Hunter and Macewicz, 1985). This could be achieved by sampling spawning fish in the laboratory (Leong, 1971); that is by taking ovarian samples at regular intervals from time of spawning, or by getting samples from a spawning aggregation over a 24 hr cycle at sea (Goldberg *et al.*, 1984; Alheit *et al.*, 1984). For the latter approach, the daily spawning cycle should be synchronous, that is, the eggs should be released at the same time interval of the day, and the average time of spawning for the population should be estimated (Hunter and Macewicz, 1985).

To estimate the number of egg batches within a spawning season, knowledge of the duration of the

individual spawning season is required, in conjunction with the spawning fraction. The duration of the individual spawning season may be estimated as the time elapsed from 50% of females in pre-spawning stages (oocytes in advanced stages of vitellogenesis previous to hydration without signs of spawning) to 50% of the females in post-spawning stages (spent and recovering) (Iles, 1964; Karlous-Riga and Economidis, 1996; Armstrong *et al.*, 2001). An important underlying assumption inherent with this approach is that the distribution pattern of the spawning stock is unchanged during the reproductive season, that is, fish do not migrate into or out of the spawning area.

Atresia

In fishes with determinate fecundity, the potential annual fecundity should be corrected for atretic losses to estimate the realized fecundity (Hunter *et al.*, 1992). Atresia is a rapid process (Hunter and Macewicz,

1985; Kjesbu *et al.,* 1991) and the amount of time from first visible signs of oocyte degeneration to complete yolk resorption (α – atretic stage) is relatively short (Fig. 6). The distinction of the later atretic stages from empty follicles becomes difficult and all studies, so far, have restricted quantification of atresia to the α – atretic (e.g., Witthames and Greer Walker, 1995). A rapid "turnover rate" of the α–stage means that a number of oocyte cohorts could degenerate from the time of advanced maturation (determination of potential fecundity) to completion of the spawning phase. As a consequence, changes in prevalence and intensity of atresia over this period could occur due to decreasing nutritional condition in the course of the individual's spawning period (Kraus, 2002). Thus, a quantification of atresia should include several estimates covering maturity stages from late vitellogenesis (determination of potential fecundity) to cessation of spawning activities.
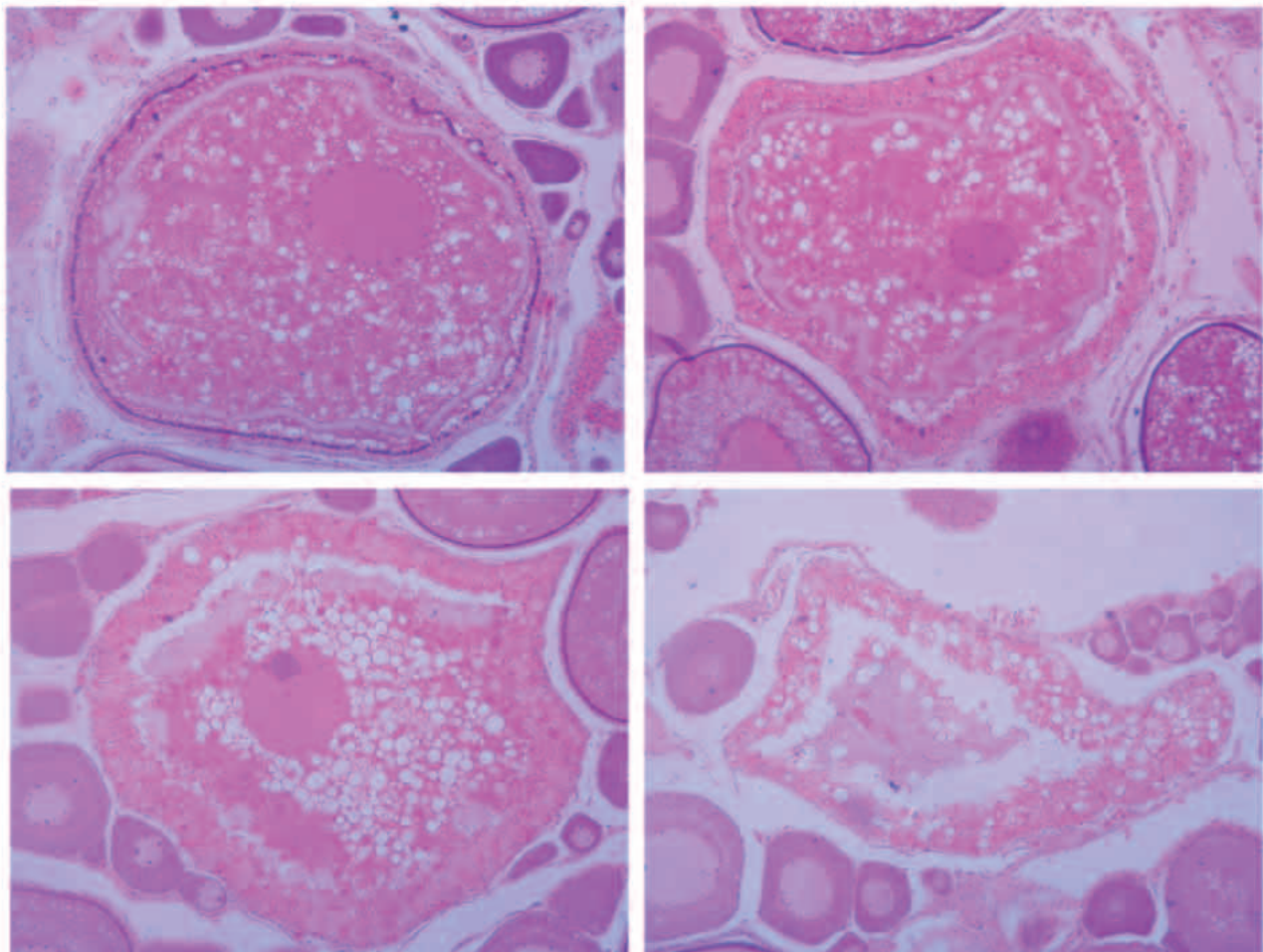

Fig. 6.   Histological sections of ovaries showing atretic oocytes at various stages of development (for further details see Appendix 2).

In contrast, the fecundity methods commonly applied to species with an indeterminate fecundity, for example, estimating batch fecundity with the hydrated oocyte or empty follicle method (Hunter *et al.*, 1985), do not need to be adjusted for egg losses due to atresia. The batch fecundity estimates refer either to fully developed oocytes ready to be spawned (hydrated oocytes) or follicles that have already released their eggs.

Several methods are available to quantify the standing stock of atretic oocytes in the ovary. However, only a few attempts to quantify atresia have been carried out. Early work (Kjesbu *et al.,* 1991) used a gravimetric method to estimate fecundity and prepared histological sections to determine the proportion of the fecundity that was atretic and hence the standing stock of atretic oocytes. This approach has been shown to be biased (Kurita *et al*., 2003) and reduced the estimated proportion of atretic oocytes to 79% compared to an unbiased stereological method (Fig. 7) based on the disector principle (applied to fish ovaries by Andersen, 2003). The problem arises because atretic oocytes shrink as they are resorbed and become

smaller than the vitellogenic group so that in two dimensional sections they are less likely to appear compared to the larger oocytes. This problem becomes even more pronounced because the smaller vitellogenic oocytes are also the most prone to become atretic (Witthames and Greer Walker, 1995). The stereometric method described above reduces the bias caused by oocyte size in three ways: i) oocyte numbers, either normal or atretic, can be estimated in three size classes based on oocyte development stage (yolk vesicle, yolk granule/yolk vesicle, and advanced yolk granule; Appendix 2); ii) it has only been applied to estimate atresia in the beginning of the alpha stage when little shrinkage in oocyte diameter has occurred; and iii) points and profiles are accumulated in separate oocyte classes. However, these assumptions have not been validated or compared with the disector method. The disector principle is considered the method of first choice as it does not require a whole cross section of the ovary. It can also be applied to species with very large ovaries such as cod or tuna because only small subsamples from the ovary are required for analysis.

Among the few attempts to quantify oocyte atresia either in natural or experimental populations, the most simple but indirect method is the comparison of potential fecundity with the realized fecundity utilizing tank experiments to collect the spawned eggs as described by Kjesbu *et al.* (1991), Bleil and Oeberst (1998), and Witthames *et al.* (MS 2000). A major problem associated with this method, however, is that the potential fecundity cannot be measured on the same individuals used to determine realized fecundity, as lethal sampling is required to remove an ovary. Consequently, the potential fecundity of reference fish of a similar size and condition has to be applied. Combining experimentally obtained total egg production rates with estimates of the intensity of atresia obtained from histological investigations, Kjesbu *et al.* (1991) estimated turnover rates of the atretic stage as 10 days. Together with information on the duration of the individual spawning season, these rates can be used to estimate total oocyte atresia from histological field samples. The experimental design was further refined in recent work by Witthames *et al.* (MS 2000) where frequent ovarian biopsies were taken during the pre-spawning and spawning period of individual captive Atlantic cod. Histological slides were prepared from these biopsy samples to determine the proportion of alpha atretic to normal developing oocytes. Using these data the production of spawned eggs and atretic oocytes could be determined during the spawning period of each experimental fish and also the average duration of
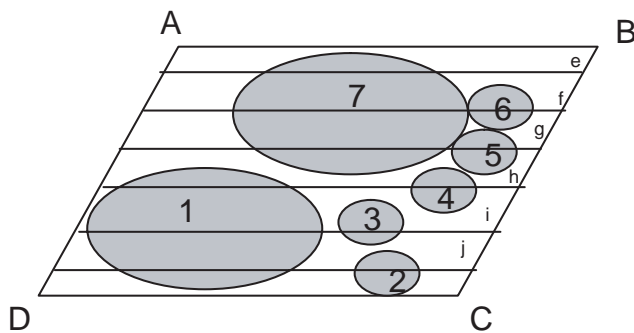

Fig. 7.   Illustration of the "disector principle" for estimating particle number in three dimensional blocks. A plain (points a–d) cuts through 7 objects; 2 large and 5 small. An object will appear in a section (parallel lines e–j) proportional to its height running from the top (A–B) to the bottom (D–C) of the plain. The ratio of small to large objects in sections e–j is 1 to 1 but the actual ratio is 1 to 2.5 (an underestimate of 2.5 times). The bias depends on the relative size of the objects. If an adjacent pair of sections are overlaid a particle is only counted if it does not appear in the 'Look up' (upper section) compared to the 'Reference' (lower section). The repeat distance between sections e–j should be 1/4 to 1/3 of the diameter of the smallest particle to function without bias; for example, particles cannot fit between sections (see Sterio (1984) and Gundersen *et al.* (1988)).

the alpha atretic stage (alpha atretic rates) could be estimated. Another method used to estimate atretic oocyte stage duration was described by Hunter and Macewicz (1985) for northern anchovy, *Engraulis mordax*. Experimental groups of anchovies were starved to initiate massive oocyte atresia followed by an intensive feeding period that led to cessation of oocyte atresia. The time elapsed until the various atretic oocyte stages disappeared was used to estimate the stage duration.

In summary, direct methods to estimate oocyte atresia require histological investigations of ovaries to determine, for example, the prevalence of individual specimens containing atretic oocytes as well as stereology (described above) to examine the intensity of atresia. To convert potential into realized fecundity, the overall atretic loss of oocytes has to be determined. This requires knowledge of atretic oocyte turnover rates that are, as part of the metabolism (among other reasons), strongly temperature dependent.

Atretic oocyte turnover rates and the duration of the individual spawning season are two of the most important items needed to quantify oocyte loss due to atresia. While the intensity and prevalence of oocyte atresia are relatively easy to obtain using stereological methods, estimates of atretic oocyte turnover rates and the duration of the individual spawning season can prove difficult. Both factors are influenced by the temperature fish experience during the pre-spawning and spawning time, as well as by their nutritional condition. In contrast to the duration of the spawning season, which is well investigated for many fish species and validated by experiments (e.g., cod: Kjesbu *et al.,* 1996), turnover rates of atretic oocyte stages have rarely been investigated (Hunter and Macewicz, 1985; Kjesbu *et al.,* 1991; Witthames *et al.,* MS 2000), and in the few studies conducted large variation was observed.

Before applying a quantitative approach we would recommend histological screening as a first step to check the relevance of oocyte atresia for the stock under investigation. Only if a significant proportion of the fish stock shows intensive atresia, is a quantitative estimation recommended. In the case of quantitative studies, not only large-scale experimental validation of both the duration of individual spawning time and atretic oocyte stages is required, but also a series of field samples covering relevant maturity stages. The large effort associated with available methods would at present hamper any quantitative routine assessment of atresia.

Estimation of Realized Fecundity

In order to estimate the annual egg production of a fish stock or population, a number of reproductive parameters have to be estimated depending on whether the reproductive modality is determinate or indeterminate. For species with determinate fecundity, the realized annual fecundity may be estimated as described in ICES (MS 1997):

$$F_{realized} = F_{potential} - F_{atresia}$$

$$F_{atresia} = I_{atr} * P_{atr} * (D/S)$$

where:

I_{atr} is the mean number of atretic oocytes per g weight of female, excluding females without oocyte atresia.

P_{atr} is the proportion of females with oocyte atresia.

S is the duration of spawning.

D is the duration of the atretic oocyte stages.

However, it should be noted again that P_{atr} and I_{atr} may vary over the pre-spawning and spawning period biasing the estimates of realized fecundity.

For species with indeterminate fecundity, the annual realized fecundity should be estimated as follows:

$$F_{realized} = BF * S * T$$

where

BF is the batch fecundity,

S is the spawning fraction,

T is the duration of spawning season.

Summary

In addition to sex ratio and proportion of mature individuals, fecundity is one of the most important determinants of a stock's reproductive potential. We have described a number of methods that could be applied to estimate fecundity for a variety of fish species. However, no universal method could be recommended, as different fecundity types (e.g., determinate and indeterminate) require specific methods to estimate the number of eggs that will be spawned per season. A variety of methods exist that could be applied to estimate the number of developing oocytes within an ovary (potential fecundity), and their application depends not only on the species under investigation, but also on the resources and laboratory facilities available. However, potential fecundity is not

always a suitable measure of reproductive potential. Potential fecundity could be a biased measure of the realized fecundity, if oocytes are continuously recruited into the pool of developing oocytes or if the number of these is reduced by atresia. Methods, so far, used to scale the potential fecundity of determinate spawners to realized fecundity, that is to account for atresia, are very time consuming and have only been applied to a few stocks (Greer Walker *et al.*, 1994; Armstrong *et al.*, 2001). In fishes with an indeterminate fecundity, none of the methods described for determinate fecundity species can be used and the estimation of batch fecundity is required.

To convert batch fecundity into realized annual fecundity, the duration of the individual spawning season and the spawning frequency have to be determined. Since the duration of the individual spawning season and the number of batches to be spawned, are variable and difficult to measure in the field, these factors represent the greatest challenge to successfully estimate the egg production of species with an indeterminate fecundity.

In conclusion, no single method could be provided to estimate the annual egg production of the variety of commercially important marine fish species. The material described here provides an overview of available methods including their strengths and weaknesses. For each attempt to quantify fecundity and total egg production, a careful review of the reproductive biology of the respective species is necessary in order to determine which method can successfully be applied.

Acknowledgements

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Appendix 1. Sampling Protocol

The sampling shall be undertaken using a commercial or research survey vessel spanning the total distribution area of the stock. Sampling and data recording should be done in the following steps:

Accessory data to be collected:

a)   Data on sample location (longitude, latitude, depth, etc.) with all basic data concerning the hauls.

b)   Length, total weight ($\pm$1g), gonad weight ($\pm$1g), gutted weight ($\pm$1g), liver weight ($\pm$1g), sex and macroscopic maturity stage.

c)   Otoliths for age determination of individuals from which ovaries have been sampled.

d)   In addition, stomach contents, liver and muscle samples (bioenergetic studies) should be carried out on females from which the gonad has been previously sampled.

Sampling for MATURITY OGIVE:

e)   A length-stratified sampling scheme for gonads and testes should be conducted during the prespawning period and around peak spawning. This sampling scheme should reflect the entire distribution area of a stock, covering both spawning and juvenile areas. The "critical" transition range from immature to mature requires a fine resolution and sufficient sample sizes to obtain reliable proportions of mature individuals.

Sampling for FECUNDITY:

For species of DETERMINATE FECUNDITY:

f)   A sample of only adult females in late vitellogenic maturity stages (stage 3) (see Table 2) should be selected whilst there are pre-spawning ripe females present in the population. All sampled fish require an identification number, weight, gonad weight, length and macroscopic maturity assignment. The sample size and the length range depends on the investigated species, but should cover the entire length range of the species. The ovary from the selected fish should be preserved in the fixative solution of choice (buffered 4% formaldehyde solution*). The fixative to ovary volume ratio should be high (4:1). The ovary membrane of big ovaries should be cut (superficially) transversally at intervals to permit penetration of fixative unless the Weibel method is applied.

For species of INDETERMINATE FECUNDITY:

g)   **_Spawning fraction_**. A random sample of only adult females in maturity stages 2–5 (see Table 2) should be collected during the spawning season. These fish represent the mature stock and are necessary for the estimation of spawning fraction of females and if possible the batch fecundity. The sample size is about 25 ovaries collected randomly from each trawl haul. The ovary from the selected fish should be preserved in the fixative solution of choice (buffered 4% formaldehyde solution*). The fixative to ovary volume ratio should be high (4:1). The ovary membrane should be cut (superficially) transversally at intervals to permit penetration of fixative.

h)   **_Batch fecundity_**. Additional sampling should be done focused to obtain hydrated females for the batch fecundity estimation. Effort should be directed to obtaining samples for batch fecundity in different parts of the spawning area. For this purpose, where spawning fraction is low, ovaries of all fish with hyaline eggs should be taken.

Preservation

Most of the fish reproductive parameters used in the fecundity analysis (spawning fraction, batch fecundity, and maturity ogive) are based on analysis of formaldehyde-preserved specimens taken with different sampling strategies. Clearly, attention has to be paid to preservation techniques with the purpose of a correct measurement of these variables.

Histological analysis requires special care in preservation. Post-ovulatory follicles are relatively subtle histological structures, and poor preservation makes it impossible to stage them. Tissue should be taken from freshly dead fish for the best preservation of morphological structure. Neither frozen specimens (or frozen gonads) nor fish dead for some time can be used (Hunter, 1985).

*The fixative should be:

100ml formalin (36% formaldehyde)
900ml distilled water
4g Na H_2 PO_4 H_2O
6g Na$_2$ HPO$_4$

Appendix 2. Histology

All the methods discussed in this review involve, at least to some extent, the histological inspection of fish ovaries. The purpose of this Appendix is to briefly present the most common procedures (for more detailed descriptions of histological procedures see Luna (1968), Preece (1972) or any histochemical handbook).

The ovaries collected for histological analyses should be handled by standard histological procedures: a cross section of about 5–7 mm is taken from the lobule and fixed/preserved in formaldehyde.

The dehydration of tissue requires moving the cross section samples through ascending concentrations of alcohol for a specific duration. Then the embedding involves transferring the cross section through ascending concentrations of resin/paraffin; and finally, the tissue samples are polymerised into resin/paraffin blocks. Subsequently, histological sections 3–5 µm thick are cut using a microtome followed by staining using the various methods as described below.

Regarding the staining, the standard procedure for Harri's Hematoxylin and Eosin is quick, easy and the cheapest standard method that most commercial laboratories offer. The results of this staining are good for identifying the different oocyte classes and the postovulatory follicles. Other stains as Mason's Trichrome, Heidenhain's iron hematoxylin, Periodic acid-Schiff reagent (PAS), PAS-Mallory Trichrome or Toluidine Blue instead of standard Hematoxylin and Eosin staining also could be applied.

Samples analyzed will be scored for presence or absence of different oocyte stages according to the following criteria (maturity ogive) and for prevalence of atresia.

Definitions of Histological Oocyte Stages. (following Wallace and Selman description, 1981) (for additional information see De Vlaming (1983); Guraya (1986); Wallace and Selman (1990); Tyler and Sumpter (1996)).

Previtellogenic oocytes

Primary Growth. This is the initial phase of oocyte growth, since the formation of the oocyte from the germinal vesicles (oogonia). It can be divided in two stages:

Chromatin nuclear. A large nucleus surrounded by a thin layer of cytoplasm. The nucleus contains a large nucleolus, and also a series of very small peripheral nucleoli. The oocyte is surrounded by a few squamous follicle cells.

Perinucleolar. Bigger nucleus with several big peripheral nucleoli. Some vacuoles appear in the cytoplasm. The chorion precursor material begins to appear in patches.

Vitellogenic oocytes

Cortical Alveoli Stage. This stage is marked by the appearance of vesicles in the cytoplasm preparatory to yolk development. The presence of cortical alveoli indicates that oocytes have started endogenous vitellogenesis. At this stage there are no eosinophilic yolk granules present in the cytoplasm. Oil vesicles begin to accumulate in the cytoplasm (if the species presents an oil globule). The chorion and follicle layers are apparent. The occurrence of this stage means that the oocyte has started the maturation process and, under normal conditions, will develop within the current breeding season.

Yolk Granule or Vitellogenic Stage. Developing yolky oocytes of large diameter with eosinophilic yolk granules present in the cytoplasm. Numerous oil droplets or vesicles should be present (if the species presents an oil globule) and the nucleus is still central.

Final maturation. The start of the final maturation phase is indicated by the migration of the nucleus to the animal pole. When the nucleus has completed its migration, the first meiotic division takes place. The hydration phase begins in many species at the end of the maturation stage, just prior to ovulation; this stage consists of a rapid uptake of fluid by the oocyte through its follicle (Fulton, 1898). This process is especially pronounced in species which spawn pelagic eggs and produce hyaline oocytes.

Post – Ovulatory Follicles

After ovulation, the structure or follicle that surrounds each oocyte collapses away from the opening formed for the release of the hydrated oocyte, and remains in the ovary as an evacuated follicle.

Early Post-Ovulatory Follicles. The structure of the follicle is very well maintained. The granulosa

and thecal epithelial layer nuclei are clearly distinguished. No signs of deterioration exist and the lumen of the POF is still open and the membranes still unfolded, i.e., very recently ovulated.

Late Post-Ovulatory Follicles. The POFs rapidly deteriorate and are resorbed, but still the organization of the POF is recognizable. Post-ovulatory follicles are recognized by the presence of rows of nuclei arranged along the axis of the degenerating follicular membranes.

Atresia

Oocytes under degeneration and oocytes that are completely resorbed. Atresia has been divided into different stages. The classification of the α-atretic stage is based mainly on the breakdown of the chorion and yolk resorption, but other changes also occur. The follicular layer becomes much more developed and the chorion appears to move in toward the center of the oocyte (Hunter and Macewicz, 1985; Witthames and Greer-Walker, 1995). At the end of the α-atretic stage, the yolk is completely resorbed and the distinction of the degenerating follicle from POF's becomes difficult. At later atretic stages the complete follicle gets resorbed. To estimate the intensity of oocyte atresia the α-atretic stage is best suited.