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SPECIAL SECTION: FISHERIES REPRODUCTIVE BIOLOGY

Estimating Oocyte Growth Rate and Its Potential Relationship to Spawning Frequency in Teleosts with Indeterminate Fecundity

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Abstract

We present a method for estimating the interspawning interval (ISI) in batch-spawning teleosts with indeterminate fecundity based on the rate of oocyte growth and the size of oocytes at the beginning and end of the spawning cycle. The method is accompanied by a number of prerequisites, which are tested and subsequently applied to wild collections of Atlantic sardine *Sardina pilchardus* (also known as European pilchard). The rate of oocyte growth and oocyte size at the end of vitellogenesis were shown to exhibit rather constant values in Atlantic sardine; thus, the ISI could be simply estimated as a factor of oocyte size at the beginning of the spawning cycle (O_b). Given that vitellogenesis in Atlantic sardine ceases at final oocyte maturation, O_b was estimated by measuring the size of oocytes of the subsequent batch in females with hydrated oocytes. The resulting average ISI was very close to inverse values of the spawning fraction estimated through the postovulatory follicle method, which indicated the validity of the ISI method. Applications of the ISI method require fewer samples of adult females compared with the postovulatory follicle method, are histology independent, and could be combined with oocyte counts to provide batch fecundity measurements. These modifications in spawning frequency and batch fecundity estimation could help to decrease both cost and labor in daily egg production method surveys.

Spawning frequency (*f*) in iteroparous fishes expresses the number of spawning events per unit time, usually per day. Spawning frequency can be estimated either as a population parameter through the spawning fraction (*S*; i.e., the proportion of females spawning per day; Parker 1980) or as an individual parameter through the interspawning interval (ISI; i.e., the time lag between subsequent spawning events; Wootton 1974). In the latter case, *f* is equal to 1/ISI. Estimates of *f* are of primary importance in exploring temporal patterns in fish reproductive dynamics (Lowerre-Barbieri et al. 2011, this special section);
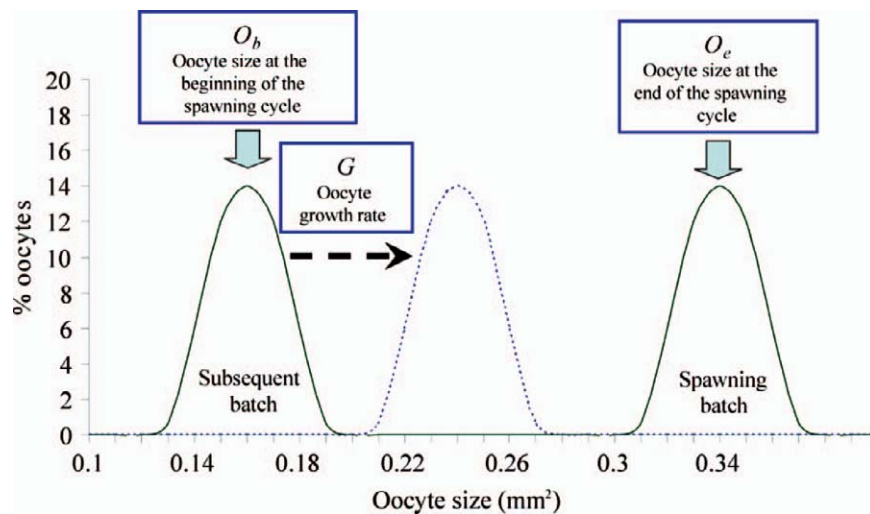
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FIGURE 1.   Conceptual model for the estimation of individual spawning frequency, exemplifying the parameters used in equation (1).

they are used in estimating spawning biomass through the daily egg production method (DEPM; Hunter and Macewicz 1985) and in calculating annual egg production (e.g., LaPlante and Schultz 2007).

To date, *f* in the wild has typically been estimated based on *S* except in species-specific applications that provide estimates of ISI, such as visual census methods (Asoh 2003; Curtis 2007), otolith microchemical analysis (Secor and Piccoli 2007), or cases where different spawning stages co-occur (e.g., co-occurrence of postovulatory follicles [POFs] of known age with oocytes at germinal vesicle migration, as in the Pacific chub mackerel *Scomber japonicus* [Dickerson et al. 1992] and European anchovy *Engraulis encrasicolus* [Uriarte et al., in press]). The most popular method for estimating *S* is the POF method, which was first introduced for northern anchovy *E. mordax* by Hunter and Goldberg (1980). Despite its popularity, the POF method can be quite inaccurate when its criteria are applied to other species and populations without prior validation and when the sampling scheme is not carefully designed (Stratoudakis et al. 2006). Furthermore, the POF method is quite costly and labor intensive because it requires large numbers of experimental fishery samples, much histology, and many work-hours from experienced personnel. Claramunt and Herrera (1994) and Claramunt and Roa (2001) presented a low-cost, histology-independent method for estimating *S* by using the gonadosomatic index as a proxy variable, whereas Zeldis and Francis (1998) estimated *S* in snapper *Pagrus auratus* by using the fraction of females with hydrated oocytes. However, in all of these cases, *S* is estimated as a population parameter, and as such it requires a large sample size, is highly subject to sampling bias, and is difficult to model in combination with certain life history variables (e.g., body size and condition) at the level of the individual. In that respect, the development of alternative methodologies that can provide estimates of *f* based on ISI seems worthwhile.

In the present paper, we introduce a method for estimating ISI in batch-spawning teleosts with indeterminate fecundity based on a conceptual model that links the rate of oocyte growth to the size of oocytes at the beginning and end of the spawning cycle (i.e., the time lag between subsequent spawning events). The conceptual model is accompanied by a number of prerequisites that are validated and subsequently applied to wild collections of Atlantic sardine *Sardina pilchardus* (also known as European pilchard) that were previously used to estimate population *S* through the POF method. Consequently, the results of our ISI method are compared and calibrated with those of the POF method. Implications for further fine-tuning of the method, its beneficial role in future DEPM applications, and its potential application to other stocks and species are also discussed.

METHODS

Conceptual model for estimating interspawning interval.—Figure 1 presents the conceptual model and illustrates the parameters needed to estimate ISI. Specifically, from knowledge of the rate of oocyte growth (G) and oocyte size (O) at the beginning (O_b) and end (O_e) of the spawning cycle, ISI can be estimated with the following formula:

$$\text{ISI} = \frac{O_e - O_b}{G}. \qquad (1)$$

Equation (1) is only valid when G is stable throughout the spawning cycle. However, most pelagic spawners undergo oocyte hydration, which is known to alter G since O increases abruptly in a very short amount of time. In that case, equation (1) should be transformed to

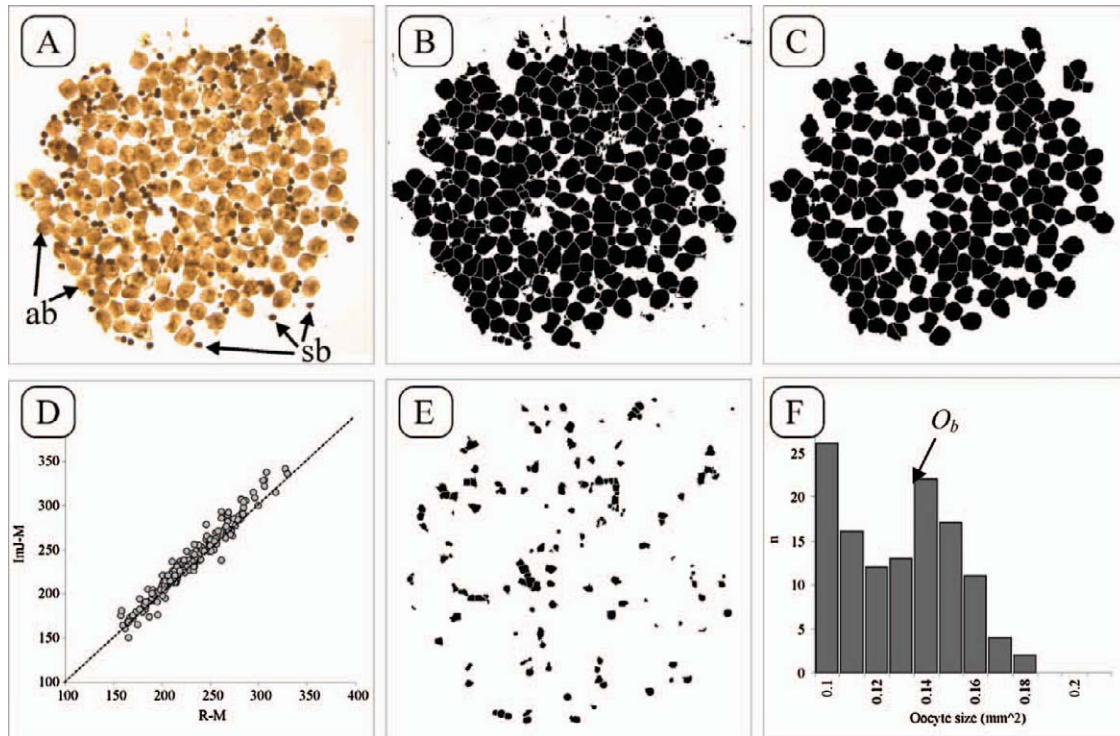$$\text{ISI} = \frac{O_v - O_b}{G_v} + \frac{O_H - O_v}{O_H} \qquad (2)$$

FIGURE 2. **(A)** Micrograph of an ovarian whole mount from a female Atlantic sardine, illustrating the oocytes of the advanced (hydrated) batch (ab) and subsequent batches (sb); **(B)** the same image after being subjected to the ImageJ routine; **(C)** separation of the spawning (hydrated) oocyte batch; **(D)** manual oocyte counts (reference method [R-M]) versus counts provided by the ImageJ method (ImJ-M) plotted over the diagonal line; **(E)** separation of nonhydrated oocytes; and **(F)** size distribution of nonhydrated oocytes, illustrating the overlap between the subsequent oocyte batch and the smaller, less-advanced oocyte groups (O_b = the modal oocyte diameter [mm^2] of the subsequent batch).

or alternatively to

$$\text{ISI} = \frac{O_v - O_b}{G_v} + t_H, \tag{3}$$

where O_v is oocyte size at the end of vitellogenesis, O_H is oocyte size at the end of hydration, G_v is the rate of oocyte growth at vitellogenesis, G_H is the rate of oocyte growth at hydration, and t_H is the time course of oocyte hydration.

To be valid, the model should satisfy the following prerequisites: (1) G_v either should be constant within the population or should be a simple function of easily measured demographic or environmental parameters like body size and temperature; (2) similarly, O_e (or O_v and t_H) should be constant or predictable based on easily measured life history or physical parameters; (3) there should be no unpredictable pauses during the spawning cycle (e.g., time lags between true vitellogenesis and hydration or "ripe holding periods"); and (4) female spawners at the beginning of their spawning cycle should be easily and accurately identified to measure O_b. Given that all these prerequisites are met and that G and O_e (or O_v and t_H) are known, ISI could be simply estimated by measuring O_b in female spawners at the beginning of their spawning cycle.

Parameter estimation and validation of prerequisites.— At each stage of oocyte development (e.g., true vitellogenesis and hydration), G can be estimated by regressing O on the spawning lag (t; i.e., the time lag before the spawning act in imminent spawners or the time lag after the spawning act in recent spawners):

$$O_i = \beta_0 + G(t_i) + e_i, \tag{4}$$

where O_i is oocyte size at spawning lag t_i, e_i is the error term, and β_0 expresses the average size of oocytes in the spawning batch at the beginning of the spawning cycle for the whole population. However, to measure O_b for each female separately, females should be caught exactly at the beginning of their spawning cycle. Based on previous findings for the Japanese pilchard *Sardinops sagax melanostictus* (also known as the Japanese sardine), the process of vitellogenesis is ceased during hydration (Matsuyama et al. 1994), implying that G in the subsequent batch also ceases while the spawning batch is growing abruptly due to hydration. In that respect, O_b can be estimated by measuring the size of oocytes of the subsequent batch in females with hydrated oocytes (Figure 2A).

The O_v can be estimated by measuring the size of oocytes of the advanced batch in a number of individuals at the transitional

TABLE 1. Characteristics of the four groups of ovarian specimens from female Atlantic sardine based on the spawning state, year of origin, preservation medium (PM; formalin or ethanol), and means of microscopic observation (MMO; histology or whole mount; n = number of specimens analyzed).

Group	Spawning state	Year	PM	MMO	n
A	Recent	2002	Formalin	Histology	125
B	Recent	2005	Formalin	Histology	68
C	Recent	2005	Ethanol	Whole mount	77
D	Recent	2008	Formalin	Whole mount	71
E	Imminent	2008	Formalin	Whole mount	278

state between the completion of vitellogenesis and the beginning of oocyte hydration. Finally, t_H can be estimated by following the time course of oocyte hydration from its very beginning to the mean daily spawning hour for the population.

Application of the interspawning interval method.— We applied the ISI method and tested each of the four prerequisites in the Atlantic sardine by use of histological specimens and ovarian whole mounts from three DEPM surveys conducted off the Portuguese coast in 2002, 2005, and 2008. Methodological details on the sampling scheme and laboratory processing of female Atlantic sardine followed in each DEPM survey are provided elsewhere (e.g., ICES 2011). In brief, each sample consisted of a random collection of 2.5–5.0 kg of Atlantic sardine, translating to a total of 50–100 individuals/sample. Immediately after capture, the fish were sexed and the ovaries from females were removed, placed in jars with formalin, and transferred to the laboratory for histological analysis (paraffin; hematoxylin and eosin). After histology, the ovaries were stored either in a 70% solution of ethanol (2005) or in formalin (2008), whereas ovaries from the 2002 survey were no longer available for further analysis in the present study.

Scoring of histological preparations included the developmental stage of the advanced group of oocytes, the presence and histological characteristics of POFs, and the prevalence and stage of atretic follicles. Subsequently, based on criteria developed by Ganias et al. (2004) for Mediterranean Atlantic sardine, females were classified as reproductively active or inactive, the former displaying at least one batch of healthy vitellogenic oocytes. Active females were further classified as spawning or nonspawning; spawning females were those showing evidence of imminent (at final oocyte maturation) or recent (presence of POFs) spawning activity.

Since the process of oocyte hydration is quite ephemeral in sardines, lasting less than 1 d in the Japanese pilchard (Matsuyama et al. 1994; Murayama et al. 1994), and because the Atlantic sardine population displays daily spawning synchronicity (Bernal et al. 2001), t for imminent spawners was estimated from the lag between the time of capture and the average daily spawning time for Iberian Atlantic sardine (2100 hours; Bernal et al. 2001). For recent spawners, t was estimated after aging their POFs by use of the procedures described by Ganias et al. (2007). Specifically, t was estimated by fitting POF cross-sectional areas to a fixed POF area–age curve and by val-

idating the estimated age with a histomorphological POF aging key.

With knowledge of t, the estimation of G_v and G_H would require measurements of O in recent and imminent spawners, respectively. Based on the year of origin, the type of fixative, the means of microscopic observation (histological slide or whole mount), and spawning state, females were classified into five different groups (Table 1); G_v was estimated in each of these groups separately by regressing O on t. Subsequently, the first prerequisite of the conceptual model (i.e., that G_v should be constant) was tested by comparing the slope of these relationships with analysis of covariance (ANCOVA).

Oocyte size in histological specimens (groups A and B, Table 1) was estimated by measuring cross-sectional areas of the three larger oocytes of the advanced batch in digital micrographs of histological preparations using ImageJ software (National Institutes of Health, Bethesda, Maryland). Oocyte size in ovarian whole mounts (groups C–E, Table 1) was measured through analysis of digital micrographs by means of automatic particle counting using a routine similar to that described by Thorsen and Kjesbu (2001). Specifically, this routine included (1) the conversion of the digital micrograph to an 8-bit image type, (2) limitation of the color spectrum to a region that included all of the oocytes (thresholding), (3) separation of individual particles (segmentation), and (4) measurement of the sizes (area) of all particles above the size order of previtellogenic oocytes (>0.030 mm^2; Figure 2A–C). This procedure proved to be very reliable since results from particle counting coincided with manual oocyte counts (Figure 2D). In many individuals, especially those that were at the beginning of the spawning cycle, the lower part of the oocyte size distribution of the advanced batch overlapped with the size distribution of the subsequent batch. For that purpose, the average size of the advanced batch was estimated through decomposing composite oocyte size distributions via a combination of Bhattacharya's method (Bhattacharya 1967) and the NORMSEP module in FiSAT II software (Food and Agriculture Organization of the United Nations, Rome, Italy; Plaza et al. 2002).

A similar procedure was applied to estimate O_b by measuring the size of oocytes in the subsequent batch for females with hydrated oocytes. The O_b was estimated only in ovarian whole mounts preserved in formalin because, as will be discussed in the next session, histology and alcohol preservation were shown
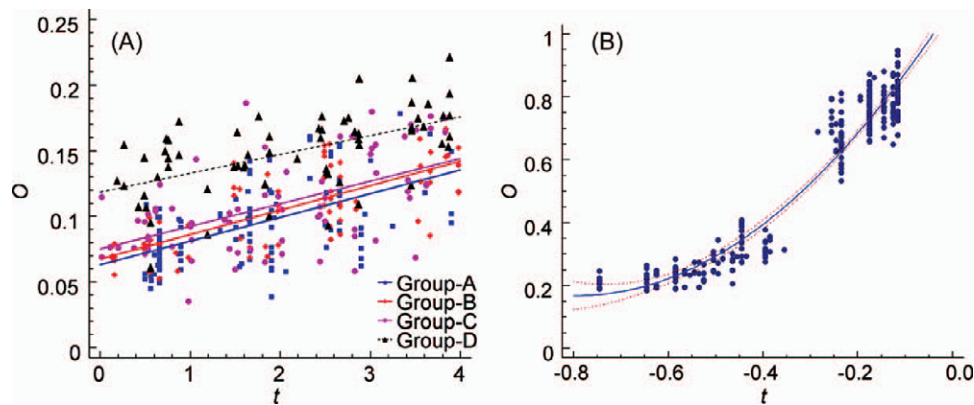
FIGURE 3. Relationship between oocyte size (O; mm^2) in the advanced batch and spawning lag (t; the time lag [d] after the spawning act in recent spawners or the time lag before the spawning act in imminent spawners) for Atlantic sardine: **(A)** four different groups of recent spawners (groups A–D, Table 1) and **(B)** imminent spawners (group E in Table 1).

to severely affect O. Therefore, the O_b and in turn the average ISI were only estimated for females with hydrated oocytes sampled in the 2008 survey. Apart from small previtellogenic oocytes, hydrated oocytes were also removed from the image analysis by counting only those particles that were below the size range of hydrated oocytes (<0.060 mm^2; Figure 2E). As was the case in the estimation of G_v, the analysis of oocyte size-frequency data had to be modified because the lower part of the size distribution of the subsequent batch usually overlapped with the size distributions of less-advanced batches (Figure 2F). For that reason, O_b in each female with hydrated oocytes was estimated again through Bhattacharya's method in FiSAT II.

RESULTS AND DISCUSSION

The ANCOVA showed that the slope of the relationship between O and t did not differ significantly between the four groups of recent spawners (groups A–D in Table 1; ANCOVA: $P > 0.10$; Figure 3A). This result suggests that G_v was constant for the 3 years of the study. On the other hand, ANCOVA revealed significant differences in the intercept of these relationships ($P < 0.001$). Multiple regression analysis showed that this difference was attributable only to group D (Table 2), indicating that histology, fixation in ethanol, or both factors caused signifi-

cant tissue shrinkage. The same analysis showed that the overall G_v was 0.018 mm^2/d (Table 2). Consistency of G_v between the 3 years becomes even more impressive considering the 50% difference in S estimates (Table 3), which corroborates evidence that variability in ISI is not induced by changes in G_v. These results seemed to satisfy the first of the model prerequisites.

Growth in O for imminent spawners is best described by the second-order polynomial regression (Figure 3B):

$$O = 0.177 - 0.010t + 0.003t^2, \tag{5}$$

where t is set in hours. This equation, together with the visual examination of Figure 3B, indicated a shift in G for imminent spawners, which was attributed (by means of histological observation) to the onset of hydration at approximately 0900 hours. Zwolinski et al. (2001) described a similar pattern for the same Atlantic sardine stock by using oocyte density data. The most important histological marker for this transition was the fusion of all yolk globules into plates. Due to the abrupt change in G at hydration, the ISI was finally estimated by use of equation (3). Consequently, these results were used to estimate both t_H and O_v. The t_H was estimated to be 12 h or 0.5 d, which is the time lag between the onset of hydration (0900 hours) and the mean

TABLE 2. Multiple regression analysis for the effect of spawning lag in the four groups of recent spawners (groups A–D, Table 1) among Atlantic sardine.

Parameter	Estimate	SE	t-statistic	P
Null	0.063	0.005	12.781	0.000
Spawning lag	0.018	0.003	6.935	0.000
Group B	0.004	0.008	0.534	0.594
Group C	0.012	0.008	1.576	0.116
Group D	0.055	0.008	6.700	0.000
Spawning lag × group B	0.001	0.004	0.197	0.844
Spawning lag × group C	−0.001	0.004	−0.210	0.834
Spawning lag × group D	−0.004	0.004	−0.958	0.339

TABLE 3. Yearly spawning fraction estimates for Atlantic sardine off the cost of Portugal. Estimates were derived from the application of the postovulatory follicle method based on either the whole female population (S) or just the population of reproductively active females (S_a). The proportion of inactive females in each S estimate is also provided.

Year	% inactive	S	S_a
1997	25	0.07	0.09
1999	6	0.10	0.11
2002	18	0.05	0.06
2005	14	0.08	0.09
2008	6	0.08	0.09

spawning time (2100 hours). The O_v was estimated by predicting O at 0900 hours based on equation (5). The value of O_v was 0.29 mm^2, which is very close to estimates for the Mediterranean population of Atlantic sardine (Ganias et al. 2004) and estimates for the Chilean sardine *Sardinops sagax sagax* (Claramunt and Herrera 1994) and the South African pilchard *Sardinops sagax ocellatus* of the Benguela Current ecosystem (Le Clus 1979). This finding satisfies the second prerequisite of the conceptual model.

Given that G_v and O_v exhibited fairly constant values and that Atlantic sardine have no pause between true vitellogenesis and hydration (Matsuyama et al. 1994) and no ripe holding period (thus satisfying the third prerequisite), ISI in females with hydrated oocytes was estimated as a factor of O_b by use of the formula:

$$ISI = \frac{0.29 - O_b}{0.018} + 0.5. \quad (6)$$

Figure 4 illustrates the distribution of ISI values for Atlantic sardine with hydrated oocytes (2008 survey only); average ISI
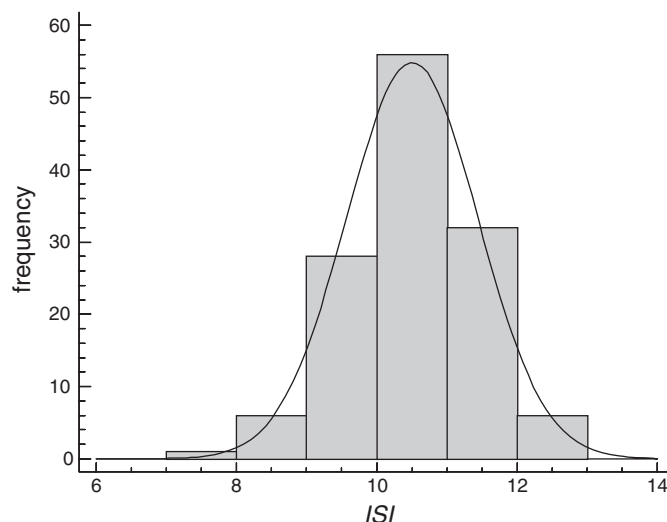

FIGURE 4. Frequency distribution of estimated interspawning intervals (ISIs; the time lag between subsequent spawning events; see equation 1) for female Atlantic sardine with hydrated oocytes; fish were sampled during the 2008 survey off the Portuguese coast. The ISI values conformed to a normal distribution (curve; $P > 0.10$).

for these females was 10.50 d (coefficient of variation = 100 × [SE/mean = 8.93%]), corresponding to an f of 0.095, which is within the range of yearly S estimates derived from the POF method (Table 3). As shown in Table 3, consistency between the two methods is improved when only reproductively active females are considered in the denominator of S estimates for the POF method. This matching between the two methods is quite encouraging for the validity of the ISI method, suggesting that if each of the conceptual model variables are carefully explored (e.g., if G is also measured in other years and in other populations of Atlantic sardine to examine possible factors that affect it), then the ISI method could be applied for estimating f both in the Atlantic sardine and possibly in other species with indeterminate fecundity.

The present findings suggest that variability in f is mostly due to variability in O_b, which is in accordance with results from histological observations that the stage or size of oocytes is quite variable for the subsequent batch in imminent spawners or for the advanced batch in very recent spawners. Furthermore, the present method allows measurements of G both during the first days of the spawning cycle and in imminent spawners prior to oocyte hydration. Although we did not provide G estimates from imminent spawners (mainly due to the limited number of samples prior to hydration), matching of the ISI method with the POF method indicates that G is constant throughout true vitellogenesis.

Apart from the aforementioned biological prerequisites, the ISI method also has certain technical prerequisites that should be met before the method is applied. Because the method's accuracy mainly depends on correct measurements of O, it must be very sensitive to several factors that affect O, like gonad preservation medium and the duration of preservation (Klibansky and Juanes 2007). In that respect, measurements of O_b and O_v may only provide comparable estimates of ISI for surveys that employ the same preservation protocols. Another important issue that must be resolved when applying the ISI method deals with the correct calibration of ISI with S estimates. As already implied, the population average of individual S estimates is equal to the average spawning interval in the population of reproductively active females. However, S estimates in DEPM surveys and in most other studies rely on the population of mature females (see Table 2 of Stratoudakis et al. 2006) and, in some cases, on the whole population, including immature fish (e.g., Rogers et al. 2003). Because at any given time active fish are a subset of the mature population, the spawning interval is overestimated (Table 2; Figure 5) and thus cannot be compared with average ISI. The only way to overcome this difficulty is to estimate S by including only reproductively active females in the denominator.

In situations where the present method is validated for a fish stock, then the f of the population can be estimated as the average ISI of females that cover the whole stock distributional area. This procedure would require fewer samples of adult females compared with the POF method, for which precision is
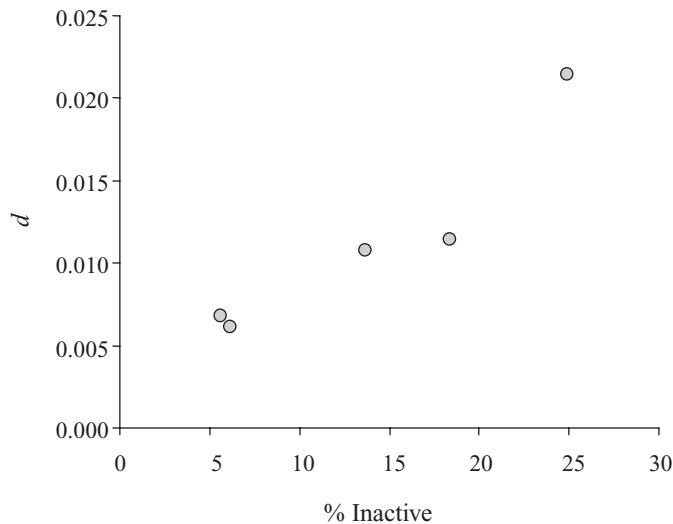
FIGURE 5.   Relationship between the proportion of reproductively inactive Atlantic sardine females and the difference (d) between the spawning fraction estimated based on the whole female population and the spawning fraction estimated based only on the population of reproductively active females.

a factor of the number of samples and the number of females analyzed per sample (Picquelle 1985). Furthermore, being histology independent, the present method would help to avoid the increased cost and labor of histological preparations and would circumvent all known problems involved with the correct aging of POFs (summarized by Stratoudakis et al. 2006). Finally, in contrast to the POF method, which should be designed in a way to avoid sampling females with hydrated oocytes and females that are very recent spawners (Stratoudakis et al. 2006), the present method would optimally work through targeting aggregations of females with hydrated oocytes. At least for Atlantic sardine, this stage is known to segregate from the remaining population at greater depths (Ganias 2008), forming layers that are detectable by means of echo-tracing (Zwolinski et al. 2006, 2007). By use of the present method, f could be easily estimated even on-board through capturing females with hydrated oocytes and instantly processing ovarian whole mounts by applying the aforementioned routine for measuring O_b. Another advantage of this procedure is that the same photomicrographs could then be used for counting oocytes of the spawning batch and for measuring batch fecundity by the procedure described in the previous section (also presented in more detail by Ganias et al. 2008). Such combined estimations of f and batch fecundity, together with the decrease in the required number of adult samples and the avoidance of histology, could help to decrease both cost and labor in DEPM surveys.

ACKNOWLEDGMENTS

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