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# Contrasting post-ovulatory follicle production in fishes with different spawning dynamics

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ABSTRACT

The assessment of postovulatory follicles (POFs) is of considerable importance when applying the Daily Egg Production Method (DEPM) since it is used to estimate both spawning frequency (S) and batch fecundity ($F_B$), provided that daily cohorts of POFs are discernible. Atlantic sardine (*Sardina pilchardus*) and Atlantic mackerel (*Scomber scombrus*), two commercially valuable pelagic species with different spawning dynamics were investigated principally to evaluate the appropriateness of the POF method to properly reflect S and $F_B$. For appropriate quantification, both the Weibel and the postovulatory follicles packing density (POFPD) method, the latter described in this paper, were applied and compared. In sardine, not only was the existence of one single daily POF cohort confirmed but also the estimated number of POFs in the cohort matched gravimetric $F_B$ results. Furthermore, the Weibel method and POFPD led to similar results. However, several daily POF cohorts co-occurred in the mackerel ovary. Therefore, POF-based estimation of $F_B$ only works for species such as sardine with a single, daily POF cohort. Likewise, in relation to estimation of S, the occurrence of single versus multiple POF cohorts makes the histological assignment of spawners to daily spawning classes either straightforward or difficult for species comparable to sardine or mackerel, respectively. In the latter case we suggest focusing in the largest, thus newest, POFs which correspond to the latest spawning event. Therefore, the appropriateness of the POF method in applications of the DEPM should be judged based on the spawning dynamics of the assessed species.

## 1. Introduction

Postovulatory follicles (POFs) are the remnants of the follicular complex after eggs are released. Their assessment is of considerable importance for the estimation of spawning stock biomass (SSB) of commercially important pelagic species using the Daily Egg Production Method (DEPM) (Parker, 1980) because they can be used to estimate spawning fraction (S), i.e., "the POF method" (Hunter and Macewicz, 1985). For that purpose, POFs must be aged (time lag between spawning and sampling) and grouped into daily cohorts to determine the number of recent spawners, whose averaged fraction in the mature population gives an estimate of S (Hunter and Macewicz, 1985; Ganias, 2013). In addition, given that POFs correspond to oocytes that are ovulated and then spawned (i.e., spawning batch) their quantification may also lead to estimation of batch fecundity, $F_B$ (e.g., Aragón et al., 2010). The latter is important when whole tissue samples are missing or discarded and (re-)calculations of fecundity can only be based on histological samples.

POF-based estimation of S and $F_B$ should depend on the spawning dynamics of the assessed species, which may affect the number of POF cohorts in the ovary. For example, species with high spawning frequency and/or slow POF resorption rates are reported to exhibit multiple POF cohorts (e.g., Haslob et al., 2003, White et al., 2003, Mackie et al., 2005, Lang et al., 2012, Mouchlianitis et al., 2020). Specifically, Mackie et al. (2005) noticed the existence of old and new POF cohorts in the ovaries of the Spanish mackerel, *Scomberomorus commerson*, as a

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result of the high spawning frequency. Additionally, Mouchlianitis et al. (2020) showed that two different POF cohorts due to two sequential daily spawning events exist in the ovaries of the Macedonian shad, *Alosa macedonica*. The authors postulated that POF degeneration was longer than the ovulatory cycle, leading to the co-existence of two POF cohorts. Because the degeneration of POFs is a continuous process, the existence of multiple cohorts with overlapping histomorphological characteristics hinders the decisive identification of distinct daily cohorts. The latter might have severe consequences for the assignment of females to the correct daily spawning class (S estimation) and for the correct enumeration of the spawning batch ($F_B$ estimation). In that respect, the ratio of the total number of POFs in the ovary (POF fecundity; $F_{POF}$) to $F_B$ should be indicative of species-specific spawning dynamics. Ratio values close to unity denote the existence of a single POF cohort whilst higher values are expected based on the number of multiple POF cohorts.

Even if POFs – mainly of earlier age and larger size - may be detected in whole mounts of ovarian tissue by means of specific staining protocols (Witthames et al., 2010), histology still constitutes the most reliable way to identify and consequently quantify the various stages of POFs (Ganias, 2013). Many fish fecundity studies use principles within stereology which lead to either assumption-based results, i.e., application of the common Weibel method (Weibel et al., 1966; Emerson et al., 1990; Medina et al., 2002, 2007; Aragón et al., 2010), results following from a series of algorithms, i.e., consulting oocyte packing density theory (OPD; Kurita and Kjesbu, 2009), or assumption-free results, i.e., application of the physical disector (Sterio, 1984). Saber et al. (2016) quantified POFs in albacore (*Thunnus alalonga*) using the latter method, which does not require any assumptions about particle shape. However, they found that the physical disector resulted in markedly higher values for batch fecundity than the Weibel and OPD methods, which matched each other well in terms of outputs.

We applied the Weibel method, strengthened and principally validated by corresponding assessments from packing density theory, to test differences in the production of daily POF cohorts in two teleosts with very different spawning frequencies: the Atlantic sardine (*Sardina pilchardus*) which spawns every 9-11 days (Ganias et al., 2011) and the Atlantic mackerel (*Scomber scombrus*) which spawns every 2-5 days (Priede and Watson, 1993). Our main working hypothesis was that $F_{POF}$ is related to S and that accumulation of multiple POF cohorts is expected for species with high S values, such as the mackerel. Multiple POF cohorts in the ovaries of the two species was examined through contrasting estimates of $F_{POF}$ from the Weibel method and the specially designed packing density theory, further validated by gravimetric $F_B$ estimates. The results from these assessments were backed-up with a detailed histological screening for the potential existence of multiple POF cohorts in the two species' ovaries. Thereafter, we considered implications of varying species-specific production rates of POFs for the proper use of the DEPM.

## 2. Materials and methods

### 2.1. Fish collection, processing and maturity staging

Ovaries from 152 sardines and 158 mackerels, all sampled in the NE Atlantic, from the Bay of Biscay to the southern North Sea, within the framework of national egg production surveys, were analysed. Sardine samples were collected in 2008 and 2014 (March to May), and mackerel samples were collected in 2013 and 2016 (February to June). Ovary weight (OW, 0.01 g), following fixation in 10% neutral buffered formalin, as well as information on fresh eviscerated body weight ($W_{EV}$, 0.01 g), were recorded.

The stored ovaries were processed both histologically, using standard procedures (paraffin embedding; 4 μm sections; haematoxylin/eosin staining), and gravimetrically (whole-mount procedures). Specimens containing POFs were selected for further scrutiny and measured for the cross-sectional area of each individual POF (POFxsa $\mu m^2$). Secondary growth oocytes were split into cortical alveoli (CA), different stages of vitellogenesis (VTG1, VTG2, VTG3), germinal vesicle migration (GVM), germinal vesicle break down (GVBD) and hydration (HYD) developmental stages (Brown-Peterson et al., 2011).

### 2.2. Quantification of POFs and batch fecundity

Given that POFs are the remnants of the follicular complex after ovulation, the number of POFs in a single daily cohort, i.e. POF fecundity ($F_{POF}$), should reflect batch fecundity ($F_B$), i.e. the number of eggs spawned in a single spawning event. Consequently, relative POF fecundity (number of POFs per gram eviscerated body weight; $RF_{POF} = F_{POF}/W_{EV}$) should equal relative batch fecundity (number of oocytes per gram eviscerated body weight; $RF_B = F_B/W_{EV}$), i.e. $RF_{POF} = RF_B$. $RF_{POF}$ was estimated using the Weibel method and specially designed packing density theory, whilst $RF_B$ was calculated gravimetrically from whole mounts.

#### 2.2.1. POF fecundity using the Weibel method

The Weibel method is based on the Delesse Principle stating that the fractional area of a particle (or group of particles) measured in a random section of a structure (e.g. tissue) equals the fractional volume ($V_i$) that the particle occupies in the same structure. A high-resolution photomosaic picture of the entire histological ovarian transverse section was reconstructed. The $V_i$ of POFs was estimated using a standard grid of 256 points within 4 counting fields of 5 $mm^2$ each following a pilot study similar to Saber et al. (2015). The number of POFs in $V_i$ ($Nv_i$) was given as:

$$Nv_i = \frac{K}{\beta} \bullet \frac{Na_i^{\frac{3}{2}}}{V_i^{\frac{1}{2}}} \quad (1)$$

where $Na_i$ is the number of POFs sectioned per unit of area, and β and K are coefficients reflecting POF shape and size distribution, respectively (Emerson et al., 1990). $Na_i$ was estimated from the same counting fields used for calculating $V_i$. Values of β and K were based on POF diameter ($POF_{DA}$) given from POFxsa. It was assumed that the shape of POFs reflected a sphere according to POF reconstructions in Korta et al. (2010) because our purpose was not to describe the exact 3-D structure of individual POFs. Mean β equaled 1.25 for both species, whereas mean K was 1.41 for sardine and 1.25 for mackerel. $F_{POF}$ was given as $Nv_i$ times ovary volume (OV). OV was estimated by dividing OW by the assumed ovarian specific gravity, set to 1.026 for both species (Ganias et al., 2015). $RF_{POF}$ was thereafter calculated (see previous paragraph).

#### 2.2.2. POF fecundity by specially designed packing density theory

The number of POFs was also calculated using the packing density theory (POFPD) following adaptations to procedures used previously on oocytes (Kjesbu et al., 2011). The number of POFs ($F_{POF}$) in the whole ovary of an individual was estimated by dividing the total volume occupied by POFs ($V_{TOT\text{-}POFs}$) by the corresponding, representative POF size ($V_{SIZE\text{-}POF}$). $V_{TOT\text{-}POFs}$ was given by multiplying OV with the volume fraction of POFs ($V_i$) (analogous to above). The largest, sectioned POF was considered as the most appropriate representative of the youngest daily POF cohort (Ganias et al., 2007). Consequently, $V_{SIZE\text{-}POF}$ was based on $POF_{DA}$ of the largest POF, correcting for shrinkage during histological processing. In the $V_{SIZE\text{-}POF}$ formula, mackerel was set to show the same level of ovarian tissue shrinkage as sardine, i.e. 51.4% (Ganias et al., 2007):

$$V_{SIZE-POF} = [(4/3) \times \pi \times (POFDA/2)^3]/(1-0.514) \quad (2)$$

The fact that sectioned POFs, in contrast to, for example, sectioned oocytes with a central nucleus, lack a proper orientation plane implied that the cutting angle was only indicative of the actual POF size. Theoretically, underestimated $POF_{DA}$ values would be expected to lead

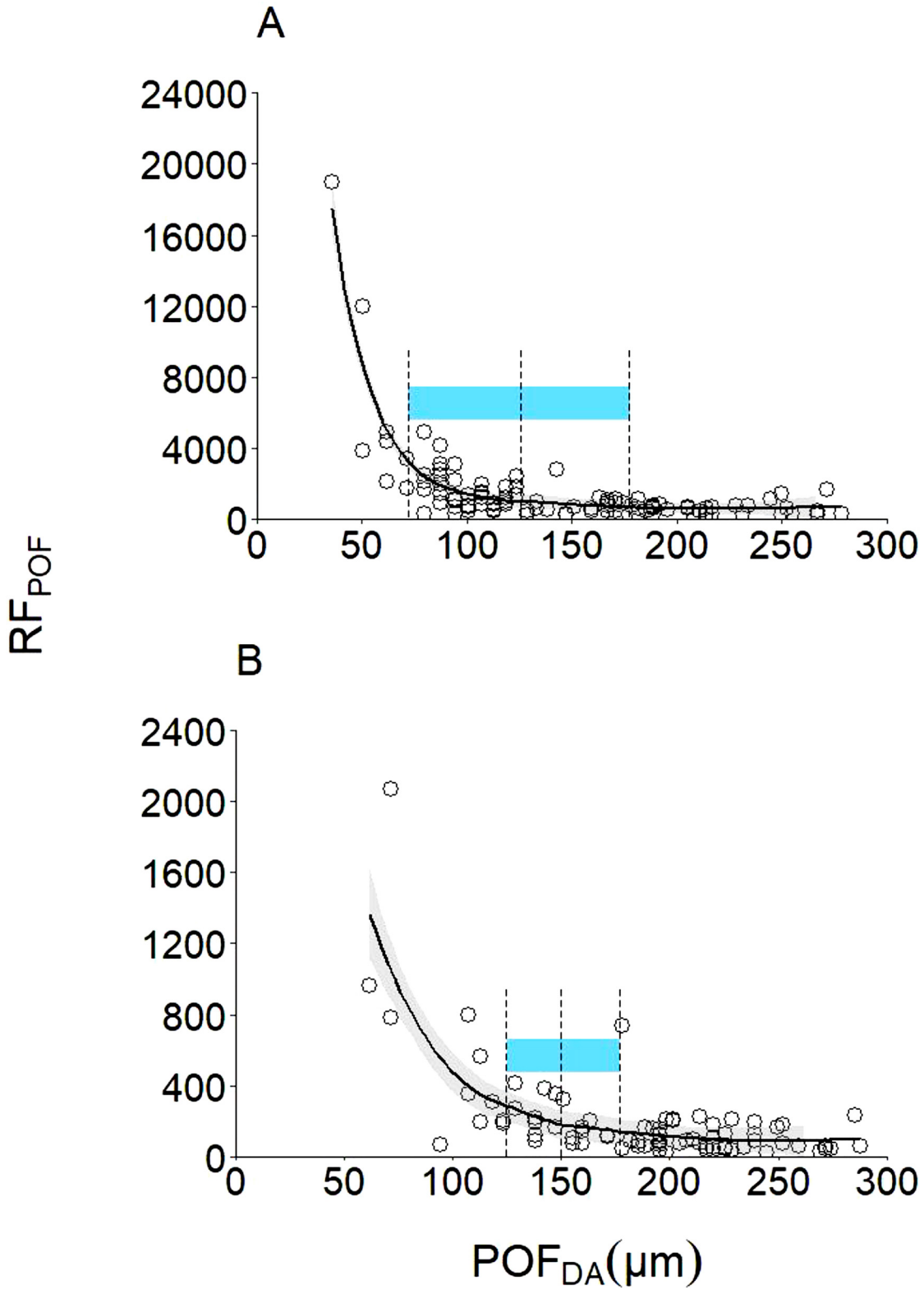


**Fig. 1.** Relationship between POF size, represented by $POF_{DA}$ (area-based POF diameter), and relative POF fecundity ($RF_{POF}$, POFs per gram eviscerated weight) for Atlantic sardine (A) and mackerel (B) using the packing density theory method. The 95% confidence interval (predicted lines with shaded area), the tested stabilization range (shaded blue area) of $RF_{POF}$, with the corresponding mathematically defined smallest, intermediate and largest values of $POF_{DA}$ inserted (vertical dotted lines) are also provided.

to inflated $RF_{POF}$ values given this methodology. To examine this potential bias, sensitivity tests were run by plotting $RF_{POF}$ for both sardine and mackerel as a function of $POF_{DA}$ (Fig. 1). $RF_{POF}$ values first dropped but then stabilized as a function of $POF_{DA}$; thus, above a critical value of $POF_{DA}$, POFs are likely sectioned through the equatorial plane representing correct size measurements. Critical $POF_{DA}$ was estimated by using the best fitted $RF_{POF}$ vs. $POF_{DA}$ functions to calculate the percent reduction in $RF_{POF}$ by successive 5 µm bins. For both species, the

percent reduction spanned from > 45% at small $POF_{DA}$ values to less than 5% at large $POF_{DA}$ values while the stabilization range for $RF_{POF}$ was found to occur for percent reduction values between 10% and 5%. Consequently, critical $POF_{DA}$ was calculated through testing variability in $RF_{POF}$ amongst the smallest ($POF_{DAS}$), the intermediate ($POF_{DAI}$) and the largest ($POF_{DAL}$) values within the stabilization range.

#### 2.2.3. Batch fecundity by the gravimetric method

Batch fecundity ($F_B$) was estimated gravimetrically using ovaries at the late VTG and GVM stages for sardine (Ganias, 2012) and at the GVBD stage for mackerel (Ganias et al., 2018). A small portion of the fixed ovarian tissue was dissected and weighed (SOW, 0.001 g), oocytes were gently separated ultrasonically and stained with toluidine blue (Anderson et al., 2020). The whole mount was captured under a stereo microscope connected with a camera (SPOT Insight Camera). In each image, the number and diameter of objects classified as oocytes in the targeted ovarian stages were recorded (Thorsen and Kjesbu, 2001). The resulting oocyte size frequency distribution (OSFD) was studied to distinguish various modes from each other and to estimate the number of oocytes of the most advanced mode ($N_{AM}$) (Ganias et al., 2010). $F_B$ was given as $F_B = N_{AM} \times OW/SOW$.

### 2.3. The number of POF cohorts

The number of POF cohorts for both species was investigated through detailed screening of histological slides for possible co-occurrence of different POF stages. This exercise was particularly complicated, mainly in mackerel ovaries due to the overlapping characteristics between the smaller POFs of the younger daily cohort(s) and the larger POFs of the next/older cohort(s). Thus, there was a continuum of POF sizes with no distinct modes, i.e. in such cases POF size could not be used in the staging of distinct cohorts as was done in previous works (e.g., Ganias et al. 2007). In addition, the fine cytomorphological aspect of individual POFs, including follicle shape and the state of the granulosa layer was often affected by methodological factors such as the cutting angle and the variable quality of histological preparations (see Ganias 2013). Consequently, our scorings only led to an approximate estimate of the number of co-occurring POF cohorts and by no means to the assignment of all the POFs from each specimen to different cohorts.

## 3. Results

The mean estimate of relative POF fecundity ($RF_{POF}$) for sardine from the Weibel grid method was 337 (95% CI = ± 48) POFs $g^{-1}$ (Fig. 2). Corresponding values from the packing density theory, modified and adopted for POFs (POFPD), were 586 (95% CI = ± 103), 346 (95% CI = ± 61) and 318 (95% CI = ± 67) POFs $g^{-1}$ (Fig. 2) when setting $POF_{DA}$ at $POF_{DAS}$ = 70, $POF_{DAI}$ = 125 and $POF_{DAL}$ = 180 μm, respectively (Fig. 1A). Only the first-mentioned $RF_{POF}$ value was significantly different (higher) (Table 1). Thus, these POFPD-based outputs for sardine stabilized from 125 μm onwards, as is also apparent from Fig. 1A. Consequently, $RF_{POF}$ at $POF_{DA}$ > 125 μm was taken as an indication of a representative POFPD-based figure. Relative batch fecundity ($RF_B$) in sardine, as estimated gravimetrically in whole mounts, was 335 (95% CI = ± 50) oocytes $g^{-1}$ (Fig. 2).

For mackerel, application of the Weibel grid method resulted in $RF_{POF}$ = 170 (95% CI = ± 23) POFs $g^{-1}$ (Fig. 2). The alternative use of POFPD, setting $POF_{DA}$ at $POF_{DAS}$ = 125, $POF_{DAI}$ = 150 and $POF_{DAL}$ = 175 μm, led to $RF_{POF}$ equal to 131 (95% CI = ± 26), 114 (95% CI = ± 26) and 110 (95% CI = ± 29) POFs $g^{-1}$, respectively (Fig. 2). No significant differences were observed amongst these values (Table 1); $RF_{POF}$ at > 125 μm was hence adopted. Contrary to sardine, $RF_{POF}$ values from the Weibel method and POFPD differed significantly (Table 1). Also, $RF_{POF}$ differed significantly from $RF_B$ outputs: mean 34 (95% CI = ± 12) oocytes $g^{-1}$ (Fig. 1; Table 1).

The histological assessment for the multiplicity of POF stages using fine cytomorphological criteria confirmed that sardines only displayed one single daily cohort of POFs in an ovary. For mackerel, this analysis showed the existence of one to up to four POF cohorts in an ovary (Fig. 3 and 4). There was a pattern of increase in $RF_{POF}$, as estimated using the Weibel method, with the number of POF cohorts (Fig. 4). Specifically, $RF_{POF}$ in females with a single daily cohort was 73.4 (95% CI = ± 34.7) POFs $g^{-1}$ which compared with the gravimetric $RF_B$ estimate despite the statistically significant difference between the two values (Welch's t-test, $p < 0.05$). Values of $RF_{POF}$ were shown to significantly increase in females with more cohorts (Kruskal–Wallis test, $p < 0.05$, followed by Wilcoxon-Mann-Whitney test applying Benjamini-Hochberg post hoc adjustment, $p < 0.05$) ending-up with an almost fourfold increase from one to four POF cohorts.

In sardine the diameter of the largest POF ($POF_{DA}$) gradually decreased from the onset of vitellogenesis, when newly formed POFs occur - just after spawning (Ganias et al., 2007) - to pre-ovulatory stages when the POFs are either very old or absent (Fig. 5A). In mackerel there was no such apparent trend, ascribed to the outlined co-occurrence of multiple daily cohorts (Fig. 5B). However, there was a clear stage-specific variation in $RF_{POF}$ in mackerel in relation to the number of co-occurring POF cohorts (Fig. 4). Specifically, 16 out of the 17 (94%) females with one or two cohorts were at VTG stages, respectively. In contrast, 22 out of the 24 (94%) of females with three or four POF cohorts were from GVM stage onwards (Fig. 4).

Given that POF size can serve as an accurate proxy for the elapsed time since egg release (see Section 1) we assumed that larger POFs were to be younger (i.e., new POFs) and thus closer to a previous spawning event. According to Ganias et al. (2007), sardines that spawned in less than 24 hrs before capture should exhibit $POF_{DA}$ values larger than 150 μm (for specimens embedded in paraffin). In the present study, and in order to target females that came from a very recent spawning event we used a range of threshold $POF_{DA}$ values above 185 μm. The fraction of females with $POF_{DA}$ above these threshold values was much higher for mackerel than for sardine. Specifically, this difference ranged from 14% for $POF_{DA}$ = 220 μm to 34% for $POF_{DA}$ = 195 μm (Fig. 6).

## 4. Discussion

### 4.1. Linking POF production to spawning dynamics

The value of $RF_{POF}$ for sardine, estimated using the Weibel and the POFPD methods, was close to both the gravimetric estimate of $RF_B$ and to the corresponding historic mean value of 384 oocytes $g^{-1}$ for Atlantic sardine stocks (Ganias et al., 2014) clearly suggesting the existence of a single daily POF cohort in this species. Contrary, $RF_{POF}$ values in mackerel were much higher than the present and previous $RF_B$ estimates for the western (31-46 oocytes $g^{-1}$; Priede and Watson 1993) and the southern Northeast Atlantic (49.6 oocytes $g^{-1}$; Ganias et al. 2018) mackerel stocks, implying the presence of more than one daily POF cohorts in this species. The differences in the number of POF cohorts was also shown through differences of POF resorption across ovarian stages in the two species.

We postulate that the existence of one single daily POF cohort in sardine should be related to its much lower spawning frequency, i.e., to the wider time window for the resorption of POFs before new ones appear. The spawning interval in the Atlantic sardine is ≈9-11 days (Ganias et al., 2011) and this relatively long spawning interval is a generalized characteristic in sardine stocks worldwide (Ganias et al., 2014). These values of S are in stark contrast to ≈2-5 days for Atlantic mackerel as reported by Priede and Watson (1993). Because the spawning interval in mackerel is markedly shorter than sardine, POFs are not anticipated to have completed the resorption process between successive egg releases, leading to the accumulation of multiple POF cohorts. A link between high spawning frequency (i.e., short spawning interval) values and multiple POF cohorts has been suggested for other fish species such Spanish mackerel, *Scomberomorus commerson* (Mackie

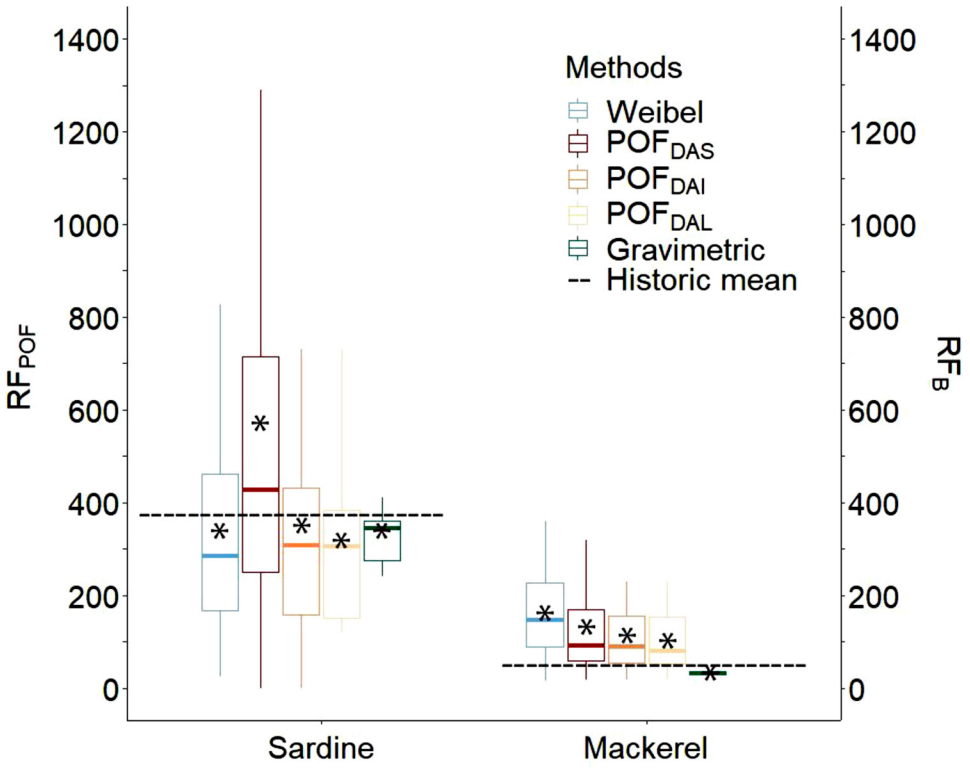


**Fig. 2.** Box and whiskers plots (Tukey style) of relative POF fecundity ($RF_{POF}$: POFs $g^{-1}$) and respective estimates of relative batch fecundity ($RF_B$: advanced oocytes $g^{-1}$) for the Atlantic sardine (left plot) and the Atlantic mackerel (right plot), using different laboratory methods: the Weibel method, the POFPD method at different POF diameter threshold values ($POF_{DAS}$, $POF_{DAI}$ and $POF_{DAL}$) and the gravimetric method. Horizontal dashed lines refer to historic mean $RF_B$ values for the two species. Asterisks correspond to arithmetic means.

**Table 1**
Statistical comparison (Wilcoxon-Mann-Whitney test applying Benjamini-Hochberg post-hoc adjustment) of estimated values of relative POF fecundity ($RF_{POF}$, POFs $g^{-1}$) estimated by POFPD and Weibel methods and relative batch fecundity ($RF_B$, advanced oocytes $g^{-1}$) estimated by the gravimetric method in Atlantic sardine and in Atlantic mackerel. Specifically, for the POFPD method $POF_{DAS}$, $POF_{DAI}$, and $POF_{DAL}$ correspond to the smallest, intermediate and largest values respectively within the stabilization range of $RF_{POF}$. *ns*: not significant difference; *: $P < 0.05$; **$P < 0.01$.

| Species | Method | $POF_{DAS}$ | $POF_{DAI}$ | $POF_{DAL}$ | Weibel |
|---|---|---|---|---|---|
| Sardine | $POF_{DAI}$ | * | | | |
| | $POF_{DAL}$ | * | *ns* | | |
| | Weibel | ** | *ns* | *ns* | |
| | Gravimetric | *ns* | *ns* | *ns* | *ns* |
| Mackerel | $POF_{DAI}$ | *ns* | | | |
| | $POF_{DAL}$ | *ns* | *ns* | | |
| | Weibel | ** | ** | ** | |
| | Gravimetric | * | * | * | ** |

et al., 2005) and the tautog, *Tautoga Onitis* (White et al., 2003). Anderson et al. (2020) suggested for Altantic cod, *Gadus morhua*, a species with very low resorption rates (Witthames et al., 2009) a gradual accumulation of recent but older POFs in the ovary during the spawning season. Mouchlianitis et al. (2020) postulated that there are multiple POF cohorts present whenever the POF degeneration progress is longer than the ovulatory cycle.

In general, scombroid fishes tend to exhibit high spawning rates; for example, 85% of female skipjack tuna, *Katsuwonus pelamis* (Hunter et al., 1986), 18%-47% of black skipjack *Euthynnus lineatus* (Schaefer, 2001), and 80% of yellowfin tuna *Thunnus albacares* (Schaefer, 1996) are reported to spawn daily. High values of S in scombroids is usually expressed by the co-occurrence of pre-ovulatory and post-ovulatory follicles (e.g., *Katsuwonus pelamis*: Hunter et al. 1986; *Scomber scombrus*: Priede and Watson 1993; *Thunnus albacares*: Schaefer 1996). Most scombroids studied so far seem to exhibit faster full POF resorption and less daily POF classes compared to other species, which might be due to the higher metabolic rates exhibited within this fish family (Dickson, 1995). In addition, scombroid fishes are unique among teleosts because of their ability to elevate their body temperature above that of water (Altringham and Block, 1997), suggesting that POF degeneration should only be partially affected by ambient temperature. We thus suggest that multiple POF cohorts in the Atlantic mackerel reflects its high spawning frequency.

The effect of spawning dynamics on the number of POF cohorts was also shown through different patterns of POF resorption across ovarian stages between the two species. Specifically, POF diameter in sardine gradually decreased from the onset of vitellogenesis, when newly formed POFs occur just after spawning (Ganias et al., 2007), to pre-ovulatory stages, when the POFs are either very old or absent, suggesting the occurrence of one cohort. In mackerel there was no such apparent trend, ascribed to the co-occurrence of multiple daily cohorts. In addition, in mackerel there was a clear pattern of increase in $RF_{POF}$, as estimated from the Weibel method, with the number of co-occurring POF stages reflecting the gradual accumulation of multiple cohorts. Thus, the existence of low or high number of POF cohorts reflects not only inter- but also intra-specific differences in the spawning dynamics. In common with sardine, mackerel with longer spawning intervals should exhibit one (or maximum two) POF cohort(s) and are thus less likely to be captured in imminent spawning stages. In contrast, females with short spawning interval should accumulate multiple POF cohorts at a rate that is proportional to the duration of full POF resorption. Given their high spawning rate, these individuals should always have their leading mode of oocytes at imminent spawning stages, i.e. from the GVM stage onwards.

### 4.2. Pros and cons of the Weibel and the POFPD methods

The sardine example shows that application of either POFPD or Weibel methods proved equally valid for species with a single daily POF cohort. $RF_{POF}$ in mackerel, was close to $RF_B$ only in a small number of individuals that seemed to display a single POF cohort. In addition, for mackerel clear statistical differences existed between the outputs of the

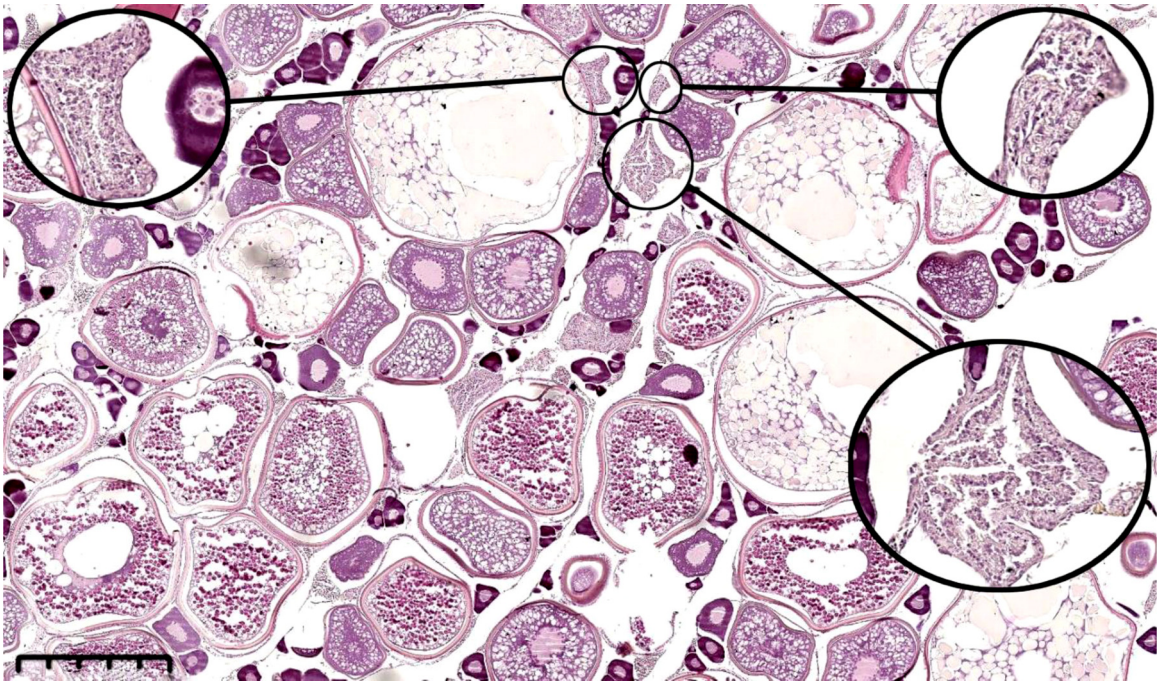

**Fig. 3.** Zooming in on Atlantic mackerel, *Scomber scombrus*, POF production. Photomicrograph of an imminent spawner with hydrated oocytes and POFs assigned to three different daily cohorts. Indicative POFs from each cohort are magnified. Scale bar: 500 μm.

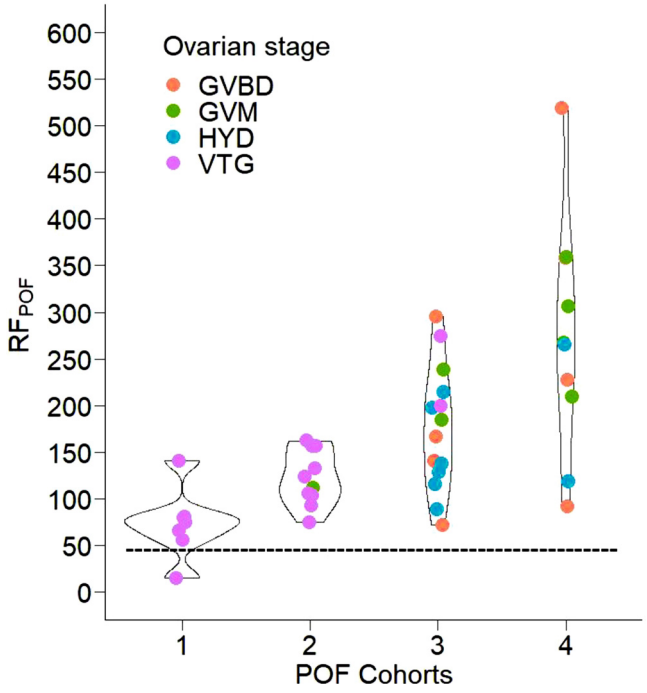


**Fig. 4.** Atlantic mackerel, *Scomber scombrus*, violin plot of POF fecundity as estimated from the Weibel method ($RF_{POF}$) for females of different developmental stages across different number of POF cohorts. VTG = all vitellogenic stages (i.e. from VTG1 to VTG3), GVM = germinal vesicle migration stage, GVBD: germinal vesicle breakdown, HYD = hydration stage. Horizontal dashed line refers to historic mean $RF_B$ values.

two methods. One apparent reason might be that the Weibel method considers all POFs included in the histological section, whereas the POFPD is based on the largest POFs only. Therefore, in case of multiple POF cohorts this latter method excludes the older POF cohorts. Consequently, $RF_{POF}$ from the POFPD method should in theory be closer to $RF_B$, as observed for mackerel. Furthermore, the typical morphometric characteristics of POFs such as the size of the lumen, the arrangement of granulosa nuclei, theca thickness, the existence of pyknotic nuclei, and the presence of vacuoles (Grier et al., 2017) can only be considered in the larger and newer POFs in each histological section. Another reason for the overestimation in POFPD estimates of $RF_{POF}$ is probably that the volume occupied by POFs, in the presence of multiple daily POF cohorts, would be inflated in terms of the representative youngest/smallest daily POF cohort volume.

When multiple cohorts of POFs co-exist it is virtually impossible in 2D (cf. histological slides) to discriminate the seemingly smallest POFs of recent cohorts from the seemingly largest POFs of older cohorts. Regarding POFPD, mathematically speaking a given measurement error (e.g. 10 μm) in POF size becomes less and less important as the true POF size increases (e.g., the volume at 110 μm is 33% greater than at 100 μm, while only 16% greater at 210 μm compared to 200 μm). This principle probably contributes to the observation that $RF_{POF}$ flattened out (reaches as stabilization plateau) with increasing POF size.

### 4.3. Implications for the DEPM

The POF method is of considerable importance in applying the DEPM as it is a popular method for estimating the spawning fraction, S (Ganias, 2013). Even though the samples in this study were collected within the framework of DEPM surveys they did not meet the requirements for an unbiased estimation of S, neither for sardine nor for mackerel. In particular, POF based estimation of S requires large numbers of samples covering the whole spawning area, whilst our samples were limited (152 sardines and 158 mackerels) and came from several areas and years. However, given that POF size can serve as an accurate proxy for elapsed time since egg release (Ganias et al. 2007; Witthames et al. 2010) we assumed that larger POFs were to be younger (i.e., new POFs) and thus closer to a previous spawning event. For the whole range of $POF_{DA}$ values, mackerel exhibited much higher fractions than sardine. This pattern was again related to differences in the spawning frequency of the two species, suggesting the validity of POF size as a proxy for estimation of S. The historical means of S (≈10% and ≈35% for sardine and mackerel respectively) were attained at a narrow range of $POF_{DA}$ between 205-220 μm (Fig. 3). This option may provide

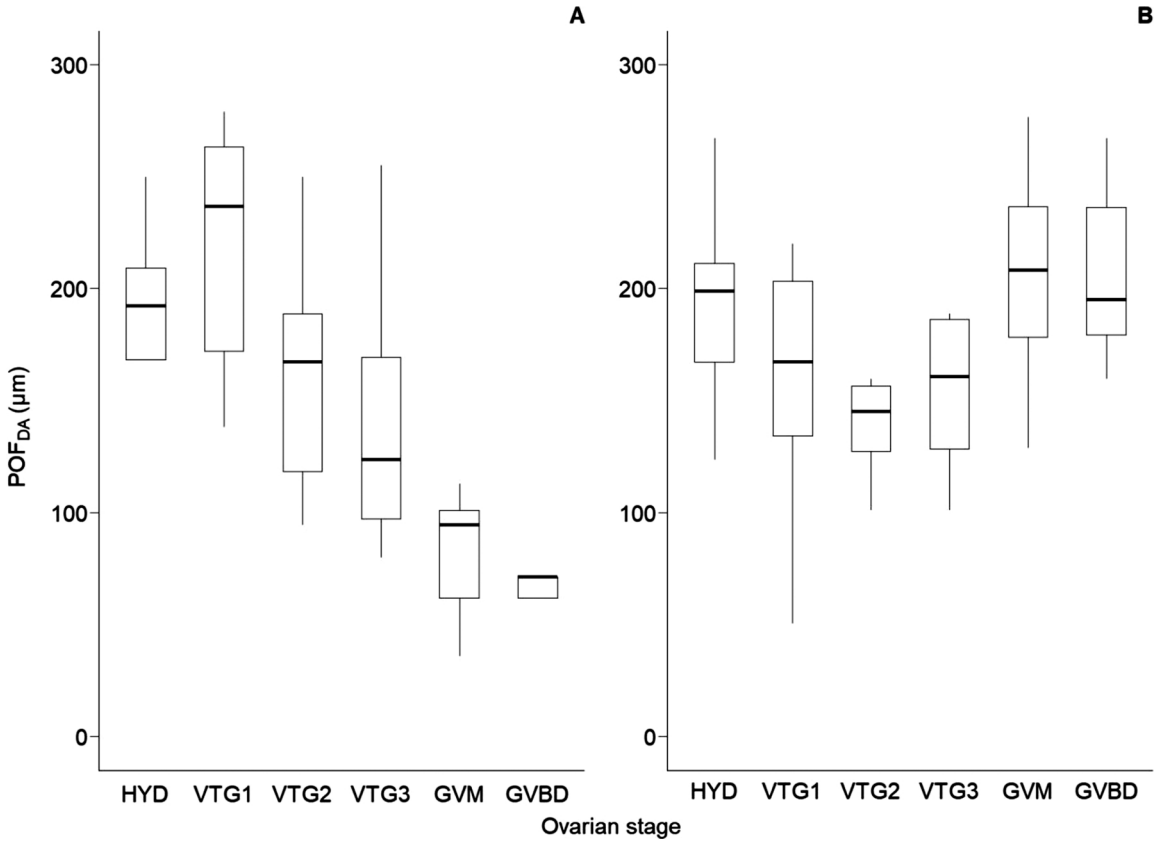


**Fig. 5.** Box and whiskers plots (Tukey style) of POF diameter ($POF_{DA,}$ µm) per developmental stage for Atlantic sardine (A) and Atlantic mackerel (B). HYD: oocyte hydration; VTG1, VTG2 and VTG3: progressing vitellogenic stages; GVM: germinal migration; GVBD: germinal vesicle breakdown.

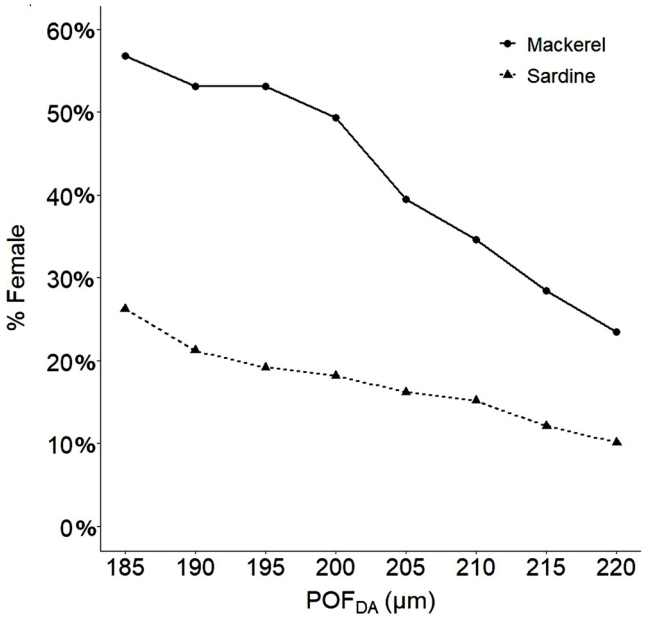


**Fig. 6.** Variation of the fraction of females with POFs above a certain threshold of POF diameter ($POF_{DA}$) for the two species.

population estimates of S focusing on the largest, thus newest, POFs and can be particularly helpful for species such as mackerel with multiple POF cohorts. Alternatively, in such species, S might be estimated at an individual level based on the assumption that the number of POF cohorts reflects differences in the spawning interval of different females. This alternative has the advantage that it requires smaller sample numbers, but it needs first to be calibrated with population estimates of S.

The present study showed that POFs might be used to reflect batch fecundity. Undoubtedly, this approach is both time-consuming, laborious and costly compared to traditional calculations of batch fecundity using the gravimetric method. However, it is quite usual in fisheries research units that whole tissue samples are discarded after some time, mainly due to storage limitations, while histological slides can be kept for decades. In such cases, (re-)calculations of batch fecundity could be based on histological screening using methods described in this paper. However, an important requirement for utilizing POFs when estimating batch fecundity is the existence of a single daily POF cohort. For instance, the existence of multiple POF cohorts in mackerel led to $RF_{POF}$ values that were much higher compared to present and historical estimates of $RF_B$. In contrast, in sardine, a species with a single clearly definable POF cohort, $RF_{POF}$ matched both our and earlier estimates of $RF_B$.

Overall, the present study demonstrates that whilst the POF method works well on species such as sardine with a single daily POF cohort, limitations are detected when applied to species such as Atlantic mackerel with several, co-occurring daily POF cohorts. Hence, the eligibility of the POF method in applications of the DEPM should be judged based on the spawning dynamics of the assessed species, especially whether single or multiple daily POF cohorts co-exist within the ovary. Such implications are of direct importance to research efforts, which intend to improve estimates of spawning frequency in fish stocks assessed by means of egg production methods (e.g., ICES, 2016).

## CRediT authorship contribution statement

**Katerina Charitonidou:** Data curation, Formal analysis, Visualization, Writing - original draft. **Olav Sigurd Kjesbu:**

Methodology, Supervision, Visualization, Writing - original draft. **Rosario Dominguez-Petit:** Funding acquisition, Writing - original draft. **Dolores Garabana:** Funding acquisition, Writing - original draft. **Maria Albisua Korta:** Funding acquisition. **Maria Santos:** Funding acquisition. **Cindy J.G. van Damme:** Funding acquisition, Writing - original draft. **Anders Thorsen:** Data curation, Formal analysis. **Kostas Ganias:** Methodology, Supervision, Visualization, Writing - original draft.

## Declaration of Competing Interest

None.

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