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ORIGINAL PAPER

# Linking the dynamic organization of the ovary with spawning dynamics in pelagic fishes

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## Abstract

The link between the dynamic organization of the ovary and spawning dynamics was investigated in pelagic fishes with indeterminate fecundity. An array of laboratory methods and statistical approaches was applied to samples of gonadal material from three commercial NE Atlantic fish species: the sardine, *Sardina pilchardus*, the horse mackerel, *Trachurus trachurus*, and the mackerel, *Scomber scombrus*. Methods included the application of clustering analysis algorithms in histological specimens and the application of particle analysis on whole mounts. More specifically, various attributes of ovarian dynamics such as the oocyte size frequency distribution, the ratio of total to batch fecundity, the number of oocyte cohorts, and the recruitment of early secondary growth oocytes were related to historic estimates of spawning interval and oocyte growth rate. It was shown that indeterminate spawners can display varying proportions of oocytes at the size range between primary and secondary growth based on the seasonal pattern of oocyte recruitment. This finding indicates that determinacy and indeterminacy should rather be recognized as end-points along a continuum which is controlled by the degree of overlap between oocyte recruitment period and spawning period. It was also demonstrated that fishes like sardine with long spawning intervals and fast oocyte growth exhibit relatively few, clearly separated oocyte cohorts, while fishes like mackerel with shorter spawning interval display increased number of coexisting cohorts. Ultimately, these aspects may provide a proxy of spawning interval and thereby spawning frequency which is a variable of paramount importance in biomass assessments of commercial fish stocks through egg production methods.

**Keywords** Oocyte growth · Oocyte recruitment · Spawning frequency · Fecundity type · Spawning dynamics

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## Introduction

To compensate for high embryonic mortality and recruitment variability, most marine teleosts maximize egg production by deploying the bet-hedging spawning strategy, i.e., shedding a series of egg batches over protracted spawning seasons (Blaxter and Hunter 1982; McEvoy and McEvoy 1992; Rochet 2000). This strategy improves the chances that at least some of the eggs or larvae will be present when 'survival windows' are open (Cury and Roy 1989; McEvoy and McEvoy 1992). Thus, it is important that oocytes are developed at the right time, so that species can breed when there is potential for reproductive success. However, the dynamic organization of the ovary—i.e., how oocytes are grouped into cohorts (Wallace and Selman 1981)—in such fishes might be exceedingly complex, as spawning capable individuals may contain numerous cohorts of oocytes from a wide range of developmental stages.

While the dynamic organization of the ovary is determined by enduring processes such as the synergistic effect of oocyte recruitment and oocyte growth (Greer Walker et al. 1994; Ganias et al. 2015), applied fisheries biology assesses spawning activity over space and time mostly based on static indicators of reproductive state, typically a measurement of gonadal development with histological analysis (Kjesbu 2009; Lowerre-Barbieri et al. 2011). Sampling individuals at one point in time—a snapshot—can be useful for classifying individuals as mature or immature, and for assessing their spawning season and spawning frequency. However, only by shifting from static indicators of reproductive state to understanding oogenesis processes such as oocyte growth rate, spawning interval, and spawning season, we can determine the fecundity pattern—determinate vs indeterminate (BOX-1)—of fish stocks and better predict how it may change with both geographic range and climate change (Hunter et al. 1985; Greer Walker et al. 1994; Schmidt-Nielsen 1983; Kjesbu et al. 1990, 1991; Witthames and Greer Walker 1995; Murua and Saborido-Rey 2003; Kjesbu 2009; Lowerre-Barbieri et al. 2011; Ganias 2013; Ganias and Lowerre-Barbieri 2018).

Ganias et al. (2015) and Ganias and Lowerre-Barbieri (2018) modelled whether and how oocyte growth rate and spawning interval affect the size frequency distribution and range of oocytes stages of individuals at sequential snapshots of the reproductive season. In temperate species, long spawning intervals and fast oocyte growth resulted in few oocyte modes with clearly separated cohorts, whereas shorter spawning intervals and slower oocyte growth increased the number of coexisting cohorts. Consequently, a wide range of different oocyte developmental stages co-occur, and at low oocyte growth and short spawning intervals, secondary growth (SG) modes begin to overlap. As these intrinsic drivers become more extreme (i.e., very short spawning intervals and slow oocyte growth), the ovary snapshot matches the description from Wallace and Selman (1981) of the asynchronous pattern where oocytes of all stages are present without dominant populations. Even though, the proof of concept for the link between the dynamic organization of the ovary and the synergistic effect of spawning interval and oocyte growth rate, hereafter labelled spawning dynamics, still needs to be established.

The objective of this paper was to investigate the above link and how spawning and ovarian dynamics shape the fecundity pattern of fishes, including the fecundity type (determinate vs indeterminate), total fecundity, and the number of co-occurring oocyte cohorts. For that purpose, three pelagic fish species with different spawning dynamics were considered: the Atlantic sardine (*Sardina pilchardus*), the Atlantic horse mackerel (*Trachurus trachurus*), and the Atlantic mackerel (*Scomber scombrus*). Concerning spawning interval, the Atlantic sardine is a sparse spawner, spawning every 10–11 d (Ganias et al. 2011), the Atlantic mackerel is a frequent spawner, spawning every 2–5 d (Priede and Watson 1993; Charitonidou et al. 2020), while the Atlantic mackerel spawns at intermediate intervals, every 3–10 d (Goncalves et al. 2009). Concerning oocyte growth rate, Ganias et al. (2015) reviewed estimates for various fish populations and showed that oocyte growth in Atlantic sardine (0.026 mm*d$^{-1}$) is one order of magnitude higher compared to Northeast Atlantic (NEA) mackerel (0.0034 mm*d$^{-1}$). For the Atlantic horse mackerel, Ndjaula et al. (2009) showed that it takes one month for the diameter of the leading oocyte cohort to grow from 214 to 284 µm which corresponds to 0.0024 mm*d$^{-1}$, which is close to NEA mackerel estimate. Accordingly, the link with spawning dynamics was investigated by evaluating various attributes of ovarian dynamics, including the oocyte size frequency distribution, the ratio of total to batch fecundity, the histological clustering of oocyte cohorts, and the recruitment pattern from primary growth to secondary growth, as expressed by the numerical ratio of early secondary growth oocytes (ESG)—on—late primary growth oocytes (LPG). These aspects were related to historic estimates of spawning interval which is a variable of paramount importance in biomass assessments of commercial fish stocks through egg production methods (Ganias 2013), especially the Daily Egg Production method (DEPM; Stratoudakis et al. 2006; Somarakis et al. 2012).

**BOX-1 traditional vs advanced assessments of fish fecundity type**

Four lines of evidence have traditionally been used to assess fish fecundity type, i.e., whether it is determinate or indeterminate (Hunter et al. 1989, 1992; Greer Walker et al. 1994; Murua and Saborido-Rey 2003; Armstrong and Witthames 2012). Briefly, these criteria include: (i) the presence (determinacy) or absence (indeterminacy) of a hiatus between primary growth and secondary growth oocytes in mature ovaries (secondary growth recruitment), (ii) the seasonal dynamics in the number and size of advanced vitellogenic oocytes, (iii) the co-occurrence of recent and imminent spawning markers, and (iv) the intensity of atresia at the end of the spawning season. Recent methodological advancements in the quantification of primary growth oocytes (Kurita and Kjesbu 2009; Korta et al. 2010; Kjesbu et al. 2011; Ganias et al. 2015; Mouchlianitis et al. 2019; Serrat et al. 2019; Anderson et al. 2020) not only have improved our general understanding of oocyte recruitment processes in both indeterminate (e.g., European hake, *Merluccius merluccius*, Korta et al. 2010) and determinate (e.g., cod, *Gadus morhua*, Kjesbu et al. 2010) spawners, but have

remarkably contributed in revising the aforementioned criteria. For instance, after their detailed assessment of primary growth oocyte groups, Serrat et al. (2019) conclude that indeterminacy in European hake is ambiguous as the pool of late previtellogenic oocyte stages corresponds to the potential annual fecundity which is a characteristic of determinate spawners. Ganias et al. (2017) used a revised set of criteria to show that horse mackerel, *Trachurus trachurus*, ceases secondary growth recruitment during the latter part of the spawning period, reflecting its controversial fecundity pattern (Karlou-Riga and Economidis 1996). The cessation of secondary growth recruitment during the spawning season has also been observed in Gulf menhaden, *Brevoortia patronus* (Brown-Peterson et al. 2017), in blueback herring, *Alosa aestivalis* (Mouchlianitis et al. 2020), and in picarel, *Spicara smaris* (Karlou-Riga et al. 2020). Schismenou et al. (2012) showed for the European anchovy, *Engraulis encrasicolus*, another indeterminate spawner, that recruitment during the ovulatory cycle occurs in pulses of very short duration, replenishing ovulated oocytes and maintaining a dynamic balance between spawned and recruited oocyte batches. Mouchlianitis et al. (2020) similarly suggested a pulsating oocyte recruitment pattern for the Macedonian shad, *Alosa macedonica*, that is triggered by the hydration of the spawning batch. All these assessments show the complexity of fish spawning dynamics that has been ignored until recently.

## Materials and methods

Samples of sardine, horse mackerel, and mackerel were collected in the NE Atlantic (off Portugal and the Bay of Biscay) at or near their spawning peak as part of national egg production surveys. Specifically, sardine samples were collected from March to May (spawning peak between March and April; Stratoudakis et al. 2007), horse mackerel samples from March to April (spawning peak between February and April; Borges and Gordo 1991), and mackerel samples from February to April (spawning peak from March to June; Olafsdottir et al. 2019). Immediately after capture, fish were sexed, females were measured for eviscerated body weight ($W_V$, 0.1 g), and ovaries were weighed (OW, 0.01 g) and fixed in 10% neutral buffered formalin.

Ovaries were processed histologically (sardine and mackerel: paraffin, 4 µm sections, Haematoxylin/Eosin staining; horse mackerel: historesin, 4 µm sections, Haematoxylin/Eosin/Safran staining). Histological sections were scanned in high-resolution micrographs using a digital slide scanner (NanoZoomer S60 Digital Slide Scanner, Hamamatsu). The ovaries were classified based on the most advanced cohort of oocytes according to Brown-Peterson et al. (2011) and those belonging to vitellogenic (VTG), germinal vesicle migration (GVM), germinal vesicle breakdown (GVBD), and hydration (HYD) stages were selected for further analysis. In total, 41 sardine, 22 horse mackerel, and 27 mackerel ovaries were used in this work.

The ovaries were also analysed through whole mount procedures. Sub-samples of ovarian tissue of 0.05–0.1 g, depending on ovarian stage, were dissected and weighed (OWs, 0.001 g). Oocytes were separated ultrasonically (Vibra-Cell VCX 130FSJ, Sonics & Materials Inc., US: 130 Watt, 50% amplitude, for 10 s; see also Anderson et al. 2020), sieved (50 µm mesh) to discard oogonia and very small primary growth oocytes and stained with toluidine blue. The whole mount was captured under a stereomicroscope connected to a camera (SPOT Insight Camera); oocyte number and diameter were measured using particle analysis (Thorsen and Kjesbu 2001) and oocyte size frequency distributions were generated.

In the oocyte size frequency distributions, primary growth (PG) oocytes highly overlapped with secondary growth (SG) oocytes. Therefore, to estimate the diameter threshold value between PG and SG oocytes, the Shazam package in R software (R core team 2020) was used (see also Mouchlianitis et al. 2020 and dos Santos Schmidt et al. 2021). The threshold values were estimated in all ovaries and the mean values of each species were set as the species-specific threshold value. Then, the relative number of primary growth oocytes ($RN_{PG}$, oocytes*$g^{-1}$) was calculated gravimetrically

$$RN_{PG} = \frac{n_{PG} \times \frac{OW}{OWs}}{W_v},$$

where $n_{PG}$ is the total number of oocytes between 50 µm and species-specific threshold value.

The recruitment pattern from primary growth to secondary growth was examined among ovarian stages of each species as well as between the three species. For this purpose, the numerical ratio of early secondary growth oocytes (ESG) to late primary growth oocytes (LPG) was used. For comparative purposes, a common size range was used for both the ESG (200–250 µm) and LPG (150–200 µm) oocytes in all three species. The size range of LPG oocytes followed pilot examinations of oocyte size frequency distributions in specimens containing only primary growth oocytes, i.e., prespawning fish and spent females (see also the study by Greer Walker et al. (1994) for mackerel). The size range of ESG oocytes was based on the findings of Ndjaula et al. (2009) for horse mackerel, assuming that there were no important interspecific differences in the size of these very early oocyte stages (see also Greer Walker et al. 1994).

The advanced mode (AM) of each oocyte size frequency distribution corresponded to the spawning batch and was considered equivalent to batch fecundity, i.e., the number of oocytes released per spawning event. Identification of the spawning batch was straightforward in oocyte size frequency distributions that exhibited a clear hiatus between the AM and the following oocytes, i.e., most sardine individuals, as well as GVBD and HYD individuals in horse mackerel and mackerel (see Results). The relative batch fecundity ($RF_B$; number of oocytes per g $W_v$) was estimated gravimetrically

$$RF_B = \frac{n_{AM} \times \frac{OW}{OWs}}{W_v},$$

where $n_{AM}$ was the number of oocytes in the AM.

Relative total fecundity of secondary growth oocytes ($RF_{SG,}$ oocytes*$g^{-1}$) was also estimated gravimetrically from whole mounts using oocytes above the species-specific diameter threshold values ($n_{SG}$)

$$RF_{SG} = \frac{n_{SG} \times \frac{OW}{OWs}}{W_v}.$$

The number of cohorts of secondary growth oocytes was estimated using three different methods: two methods based on whole mounts and one on histological specimens. The first method was the 'fecundity ratio' calculated as the ratio of $RF_{SG}$ to $RF_B$ (Ganias et al. 2017; Mouchlianitis et al. 2020). The second method estimated the number of cohorts of secondary growth oocytes from histological specimens by applying the clustering analysis algorithms to the characteristics of oocyte histological structures as described in the study by Charitonidou and Ganias (2021). In particular, the oocytes of each ovary were distinguished into two groups, the first group contained oocytes with well visible and measurable cortical alveoli (*ca*) (oocyte group I, OcGI), while the second group included well visible and measurable yolk granules (*yg)* (oocyte group II, OcGII). Subsequently, the maximum size ($ca_{max}$ or $yg_{max}$) and the relative area, namely the percentage of the area (intensity) occupied by the ca ($ca_{int}$) or yg ($yg_{int}$) in the oocyte cytoplasm, were measured by image analysis procedures described in Charitonidou and Ganias (2021). Oocytes were classified into cohorts by implementing K-means clustering analysis using the Stats package in R software (R core team 2020). This procedure was performed for oocytes up to the end of VTG stage. The advanced oocyte mode from the GVM stage onwards was considered as a single, well-distinguishable cohort, which was subsequently manually added to the number of cohorts calculated by the clustering analysis (Charitonidou and Ganias 2021).

The third method was based on the analysis of oocyte size frequency distributions (obtained from whole mounts) and the decomposition of overlapping oocyte size distributions (Bhattacharya et al. 1967) to identify the number of different secondary growth oocyte modes. In addition, the relative fecundity of each oocyte cohort (cohort specific fecundity, $RF_{ci}$) was assessed gravimetrically as described above.

All statistical analyses were performed in R software (R core team 2020). Data analysis was performed using the dplyr package. Visualization of the data was performed using the packages ggplot2, ggpubr, and factoextra. The plots of oocytes size frequency distribution were visualized using the Ggjoy package. Normality of data was tested using the Shapiro test. Pairwise comparisons with corrections for multiple testing were performed. Specifically, for parametric data, one-way ANOVA test was implemented, and if the results were significant, the Tukey's test was applied. For non-parametric data, the Kruskal–Wallis test was applied, and when results were significant, a Pairwise Wilcoxon Rank Sum Test was performed in conjunction with the Benjamini–Hochberg post hoc adjustment.

## Results

The threshold values between primary growth and secondary growth oocytes were quite close among the three species: 174.6 (±3.5) µm for sardine (Fig. 1a), 176.8 (±6.6) µm for horse mackerel (Fig. 1b), and 197.4 (±7.4) µm) for mackerel (Fig. 1c), even though the threshold in mackerel was significantly higher (ANOVA: $p < 0.05$). These statistically given thresholds based on oocyte frequency distributions (OSFDs) of primary growth (PG) oocytes were incorporated in the further estimation of the relative number of PG oocytes ($RN_{PG}$), the relative total fecundity of secondary growth (SG) oocytes ($RF_{SG}$), and to construct the OSFDs of SG oocytes for each species. The mean $RN_{PG}$ was 2474 (±390) oocytes*$g^{-1}$ for sardine, 2108 (±459) oocytes*$g^{-1}$ for horse mackerel, and 3409 (±1335) oocytes*$g^{-1}$ for mackerel (Fig. 2). The mean $RN_{PG}$ of horse mackerel in the VTG stage was lower than for the two other species, but this difference was non-significant (ANOVA: $p > 0.05$) (Fig. 2). Also, the $RN_{PG}$ values showed higher variances in the GVM stage in sardine, in the GVBD stage in horse mackerel, and in all stages in mackerel (Fig. 2).

In all three species, the oocyte size frequency distribution was continuous in the size range between 150 and 250 µm indicating continuous recruitment of secondary growth oocytes during all reproductive stages (Fig. 1). However, the shape of the distributions differed, particularly between sardine (smooth pattern, Fig. 1a) and mackerel (highly skewed pattern, Fig. 1c), while the pattern

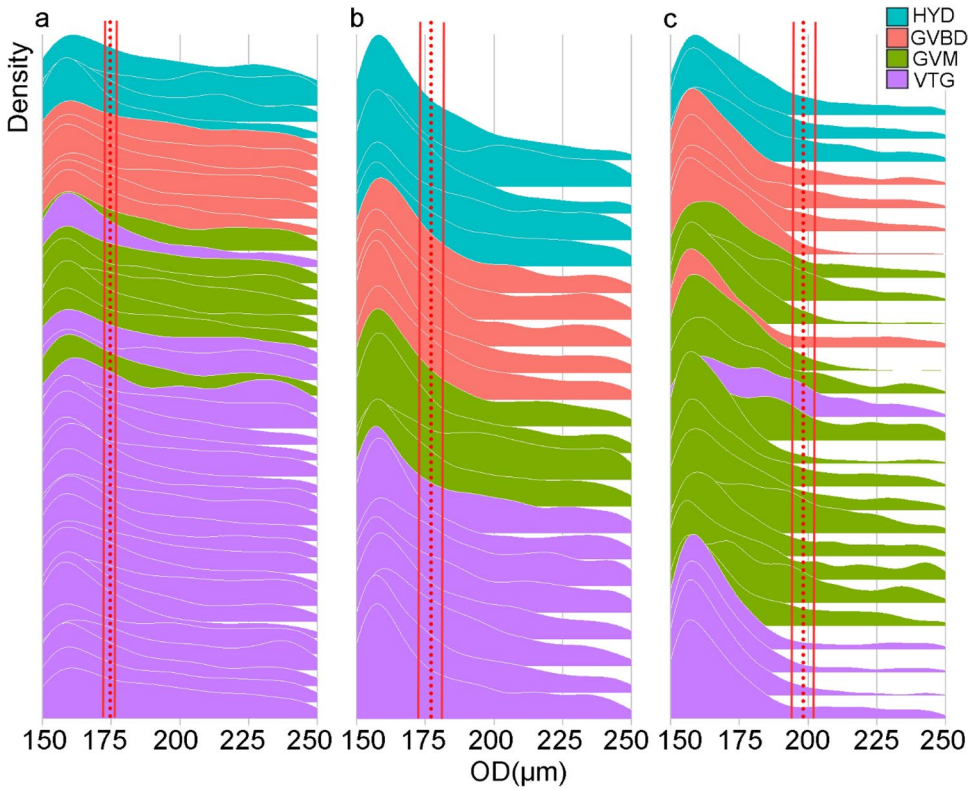


**Fig. 1** Frequency distributions of oocytes with diameters between 150 and 250 µm (OD, µm). Rows represent different individuals in ascending order of maximum oocyte size observed within the ovary: **a** sardine (*Sardina pilchardus*), **b** horse mackerel (*Trachurus trachurus*), and **c** mackerel (*Scomber scombrus*). Colours indicate ovarian stages, vitellogenic stage (VTG, purple), germinal vesicle migration (GVM, green), germinal vesicle breakdown (GVBD, pink), and hydration stage (HYD, blue). Mean threshold diameter values between primary growth and secondary growth oocytes are indicated by the vertical red dashed lines. Solid lines: 95% confidence intervals

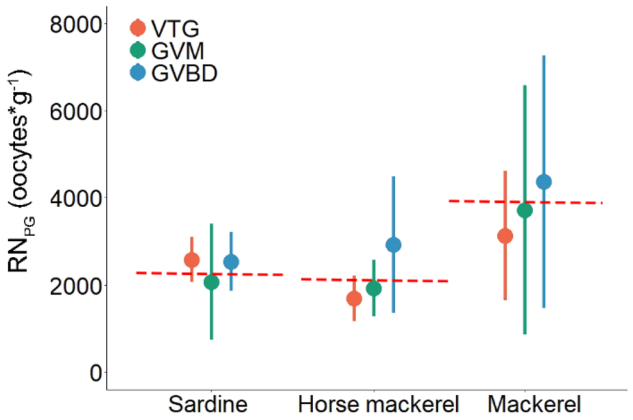


**Fig. 2** Relative number of primary growth oocytes ($RN_{PG}$, oocytes*$g^{-1}$) per ovarian stage (VTG: vitellogenesis; GVM: germinal vesicle migration; GVBD: germinal vesicle breakdown stage) for sardine (*Sardina pilchardus*), horse mackerel (*Trachurus trachurus*), and mackerel (*Scomber scombrus*). Filled circles: mean value of $RN_{PG}$ at each ovarian stage; bar: 95% confidence intervals; horizontal dashed lines: mean $RN_{PG}$ value of all ovarian stages in each species

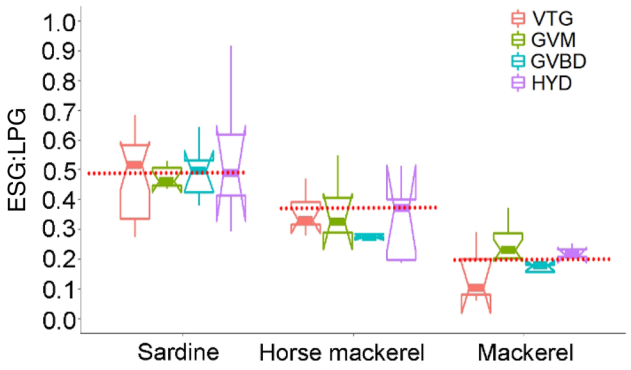


**Fig. 3** Box and whiskers plots of the numerical ratio of early secondary growth to late primary growth oocytes (ESG:LPG) per ovarian stage for sardine (*Sardina pilchardus*), horse mackerel (*Trachurus trachurus*), and mackerel (*Scomber scombrus*). *VTG* vitellogenic stage, *GVM* germinal vesicle migration, *GVBD* germinal vesicle breakdown stage, *HYD* hydration stage. The median is marked by the thick line within each box and the mean ratio value of each species is indicated by the dashed line

in horse mackerel was intermediate (Fig. 1b). These differences lead to significant ($p < 0.005$) differences in the numerical ratio of early SG oocytes (200–250 µm) and late PG oocytes (150–200 µm), i.e., ESG:LPG, between the three species. In particular, the ESG:LPG ratio was significantly higher ($p < 0.05$) in sardine ($0.48 \pm 0.04$) compared to horse mackerel ($0.33 \pm 0.04$) and mackerel ($0.19 \pm 0.04$). On the other hand, within each species, this ratio did not differ significantly between the different ovarian stages ($p > 0.05$; Fig. 3). Nevertheless, in horse mackerel, the ESG:LPG ratio in GVBD was apparently lower compared to VTG and GVM, and in mackerel, a difference

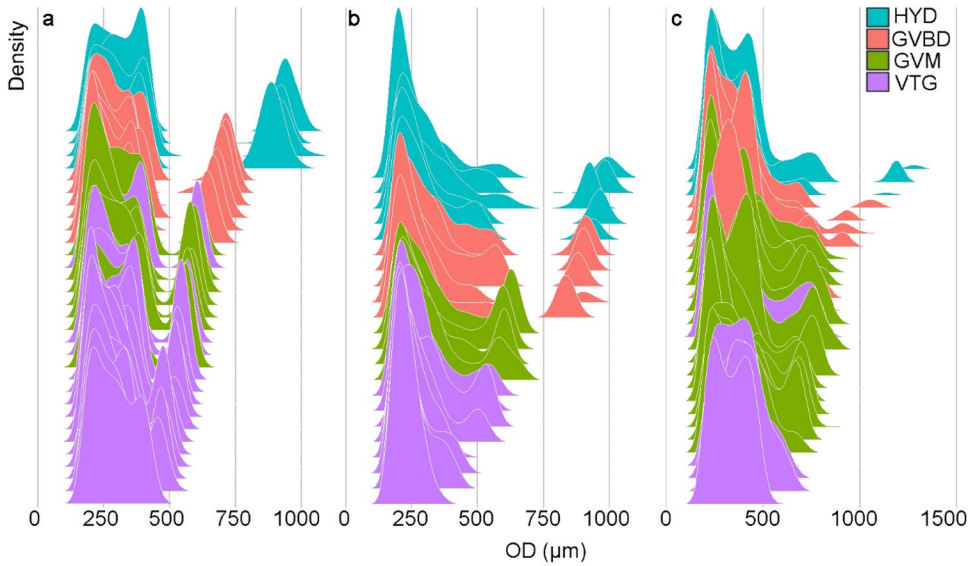


**Fig. 4** Oocyte size frequency distributions (diameter > 150 µm) per ovarian stage for the three assessed species: **a** sardine (*Sardina pilchardus*), **b** horse mackerel (*Trachurus trachurus*), and **c** mackerel (*Scomber scombrus*). Rows represent different individuals of the three species in ascending order of maximum oocyte size. *VTG* vitellogenic stage, *GVM* germinal vesicle migration; *GVBD* germinal vesicle breakdown stage, *HYD* hydration stage. Picking joint bandwidth was 15.3 for sardine, 7.15 for horse mackerel, and 9.44 for mackerel

was noticed between the VTG and the other ovarian stages (Fig. 3). In both species, these GVBD and VTG values were close to the mean ratio of each ovarian stage, without high variances (Fig. 3).

The oocyte size frequency distribution of secondary growth oocytes in all three species was always continuous and polymodal (Fig. 4). The only clearly distinguishable mode was the advanced one (AM), which grew progressively and, at some point, was separated with a size hiatus from the intermediate secondary growth oocytes—i.e., those between the primary growth–secondary growth threshold and the AM hiatus—hereinafter referred to as the "SG-pool". In sardine, the hiatus between the SG pool and the AM occurred at the early VTG stage (Fig. 4a), whereas in horse mackerel and mackerel it occurred later at the GVBD and GVBD-HYD stages, respectively (Fig. 4b, c). The maximum oocyte diameter of the SG pool reached 450–500 µm, but never exceeded 500 µm in sardine (Fig. 4a), 600–750 µm in horse mackerel (Fig. 4b), and 700–850 µm in mackerel (Fig. 4c). Most ovaries in sardine and mackerel were at the VTG and GVM stages, respectively, while horse mackerel again showed an intermediate pattern between the other two species (Fig. 4).

The number of secondary growth oocyte cohorts estimated through the fecundity ratio in whole mounts and the clustering analysis in histological specimens did not differ significantly between the two methods, neither in sardine [5 (±0.4) and 4.8 (±0.3), respectively; $p > 0.05$], nor in horse mackerel [5.8 (±0.8) and 6.4 (±0.4), respectively; $p > 0.05$] (Fig. 5a,b). However, for mackerel, the results were significantly different between the two methods: 14.6 (±2.5) modes were estimated through the fecundity ratio and 10.2 (±2.2) through the clustering analysis ($p < 0.05$) (Fig. 5c).

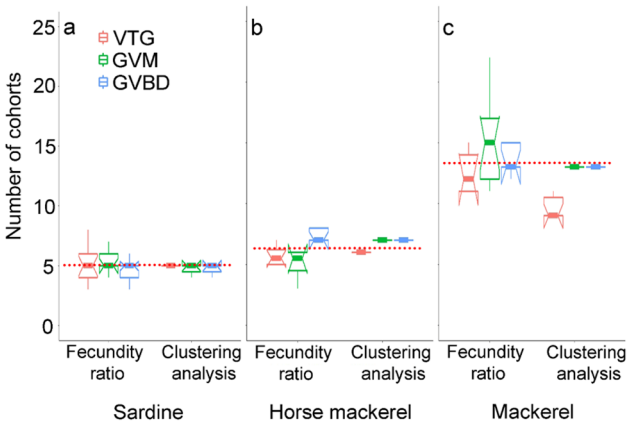


**Fig. 5** Box and whiskers plots of the number of oocyte cohorts estimated by the fecundity ratio and K-means clustering analysis at each ovarian stage, vitellogenic stage (VTG), germinal vesicle migration (GVM), and germinal vesicle breakdown (GVBD), for the three species **a** sardine (*Sardina pilchardus*), **b** horse mackerel (*Trachurus trachurus*), and **c** mackerel (*Scomber scombrus*). The median is marked by the thick line within each box and the general mean value (including all ovarian stages) of the number of modes estimated by the fecundity ratio of each species is indicated by the dashed horizontal line

In all three species, the fecundity ratio was not significantly different between ovarian stages ($p > 0.05$) (Fig. 5). Regarding the clustering analysis results, there was no statistical difference in the number of cohorts between the ovarian stages for sardine (Fig. 5a); however, for horse mackerel, the VTG stage was found to contain a significantly lower number of modes than the other two stages ($p < 0.05$) (Fig. 5b). Similarly, in mackerel, the number of modes was statistically lower between the VTG stage [8.8

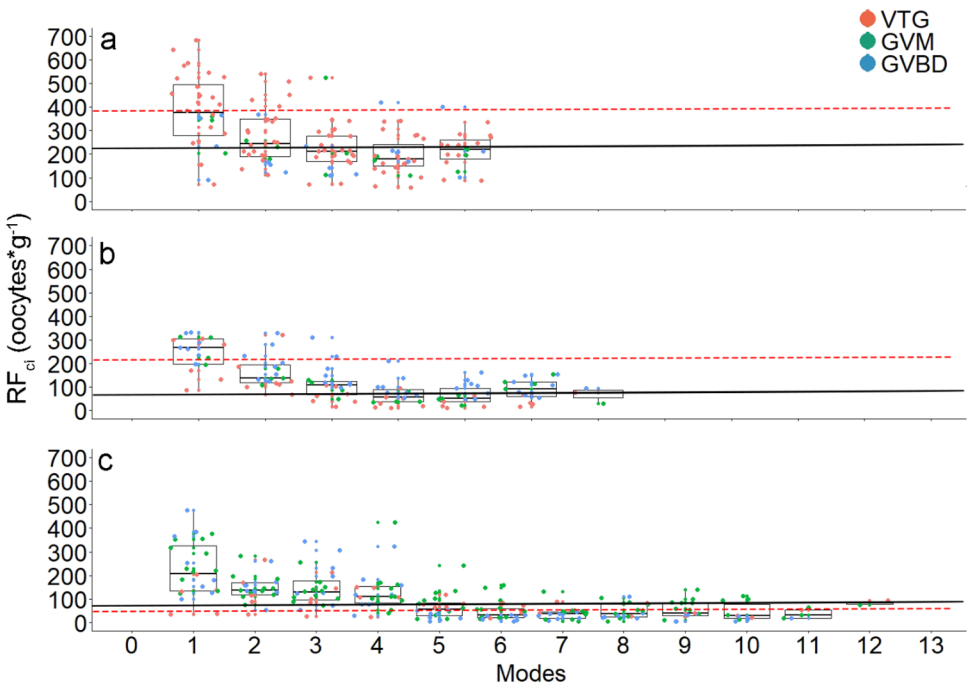


**Fig. 6** Box and whiskers plots of relative cohort fecundity values ($RF_{ci}$, oocytes*$g^{-1}$) as estimated for each mode from the decomposition of overlapping oocyte size distributions for each species: **a** sardine (*Sardina pilchardus*), **b** horse mackerel (*Trachurus trachurus*), and **c** mackerel (*Scomber scombrus*). The median $RF_{ci}$ value is indicated by the dashed horizontal lines. The solid horizontal lines indicate the mean values of advanced mode relative batch fecundity ($RF_{AM}$, oocytes*$g^{-1}$) for each species. The ovarian stages are indicated by different colours: *VTG* vitellogenic stage, *GVM* germinal vesicle migration, *GVBD* germinal vesicle breakdown stage

(±2.7)] and both the GVM (13) and GVB ovarian stages (13) ($p < 0.05$) (Fig. 5c).

Decomposition of overlapping oocyte modes provided an estimate of cohort specific fecundities ($RF_{ci}$) (Fig. 6). These estimates were contrasted with the mean $RF_{AM}$ values for each species. For all three species, independently of ovarian stage, almost all oocyte cohorts showed stable mean $RF_{ci}$ values that were very close to $RF_{AM}$ values. Only cohorts of smaller oocytes, i.e., cohort #1 in sardine, cohorts #1 and #2 in horse mackerel, and cohorts #1 to #4 in mackerel, showed significantly higher ($p < 0.05$) mean $RFc_i$ values compared to $RF_{AM}$ and to the remaining cohorts (Fig. 6). Also, mean $RF_{AM}$ between ovarian stages did not differ significantly ($p < 0.05$).

## Discussion

The present study revealed similarities, but also important differences in the dynamic organization of the ovary among the three studied species. These differences were linked to respective differences in oocyte recruitment, spawning interval, and oocyte growth rate. The distribution of smaller oocytes in the size range between late primary growth (LPG) and early secondary growth (ESG) was continuous in all three species, indicating continuous secondary growth recruitment. This pattern is a generic characteristic of indeterminate spawners, where oocyte recruitment is an ongoing process within the spawning season (Hunter et al. 1985; Greer Walker et al. 1994; Murua and Saborido-Rey 2003; Armstrong and Witthames 2012). Nevertheless, recent studies show that determinate spawners like *Gadus morhua* can also display continuous oocyte size frequency distribution (Anderson et al. 2020), while according to Ganias (2013), pure determinacy and indeterminacy are rather recognized as end-points along a continuum of intermediate patterns which is generally controlled by the time lag between oocyte recruitment period and spawning period. In that respect, the continuity between primary growth and secondary growth oocytes is not always indicative of the fecundity type and other aspects such as the characteristics of oocyte size frequency distribution should also be considered (Hunter et al. 1989, 1992; Kjesbu et al. 1990; Witthames and Greer Walker 1995; Ganias 2013). Also, complexities in the seasonal spawning pattern such as the cessation of oocyte recruitment at the latter part of the spawning period also need to be considered (see BOX-1; Brown-Peterson et al. 2017; Ganias et al. 2017; Mouchlianitis et al. 2020; Karlou-Riga et al. 2020).

There were remarkable differences in ESG:LPG ratios between the three species. The highest values were observed in sardine and the lowest in mackerel, while horse mackerel displayed intermediate values. These findings apply to the peak of the spawning period; however, the situation may be different at the onset or end of the spawning period (see also BOX-1). It is thus proposed that the ESG:LPG ratio can serve as a proxy of secondary growth recruitment dynamics mostly reflecting the annual fecundity pattern of a fish population/species. In particular, species like sardine with continuous SG recruitment throughout the spawning period

(Ganias et al. 2014), tending to the indeterminate fecundity end-point, should display higher ESG:LPG ratios. On the other hand, horse mackerel was previously shown to cease recruiting secondary growth oocytes at the latter part of the spawning season (Ganias et al. 2017); this pattern is untypical for indeterminate spawners, consequently leading to a lower ESG:LPG ratio. In mackerel which displayed the lowest ESG:LPG ratio among the three species, the tapering of secondary growth recruitment should be even more pronounced. Indeed, Greer Walker et al. (1994) showed a steep decrease in the proportion of tiny oocytes (120–144 µm) with ovarian development, suggesting a tapering in oocyte recruitment with the progress of the reproductive period (see also the study of Mouchlianitis et al. (2021) for the blueback herring, *Alosa aestivalis*). Even if both mackerel and horse mackerel are now considered as indeterminate spawners (Macer, 1974; Karlou-Riga and Economidis 1997; Gordo et al., 2008; Ndjaula et al., 2009; ICES 2011, 2012, 2018; Ganias et al. 2017; Jansen et al. 2021; dos Santos Schmidt et al. 2021), their differences in ESG:LPG ratio indicates tendency towards the 'determinate' end-point; this explains why the fecundity pattern of these two species has been so controversial during the last decades (see also BOX-1). We may thus conclude that even if oocyte growth rate and spawning interval affect the recruitment rate from LPG and ESG, the degree of overlap between the two oocyte groups is mostly regulated by the annual fecundity pattern, i.e., the time lag between the oocyte recruitment period and spawning period. Given that both spawning interval and oocyte growth rate are influenced by ambient temperature [see reviews by Ganias (2013) and Ganias et al. (2015)], we may suppose that ESG:LPG ratio can also be temperature-dependent.

On the other hand, it is suggested that the size frequency distribution of secondary growth oocytes is mostly shaped by the spawning interval and the oocyte growth rate. In particular, the present study showed that the ovarian stage where the size hiatus between the advanced oocyte mode and the SG pool was established differed between the three species. In sardine, the spawning batch was separated from the SG pool since mid-vitellogenesis. A similar pattern is also reported for two other clupeids (*Etrumeus teres*: Plaza et al. 2007; *Etrumeus golanii*: Somarakis et al. 2021) in which the advanced batch grows rapidly after spawning and shortly separates from the smaller oocytes. In horse mackerel and mackerel, this separation took place at various phases of final oocyte maturation (FOM). In addition, there were important differences in the size frequency distribution and the size range of the SG pool. In sardine, the SG pool consisted of a small number of oocyte cohorts that were always up to the early VTG stage. In mackerel, the SG pool consisted of a high number of oocyte cohorts which reached the final stages of vitellogenesis and the beginning of FOM, while horse mackerel displayed an intermediate number of cohorts. As reviewed in the Introduction, sardine spawns at longer intervals (every 10–11 days), horse mackerel at intermediate intervals (every 3–10 days), while mackerel is a frequent spawner, spawning every 2–5 days. The remarkable difference in spawning frequency between sardine and mackerel was also reflected in the differential production of post-ovulatory follicles between the two species in Charitonidou et al. (2020).

The present results provide support for the conceptual model of Ganias et al. (2015) and Ganias and Lowerre-Barbieri (2018), who simulated oocyte size frequency distribution in multiple spawners based on oocyte growth, spawning interval, and the duration of the spawning period. Based on this modelling approach, sparse spawners with faster oocyte growth rate display polymodal oocyte size frequency distributions consisting of fewer and distinct oocyte cohorts, while frequent spawners with slower oocyte growth rate tend to form a unimodal distribution consisting of numerous oocyte cohorts which are hardly distinguished from each other. Indeed, the number of estimated secondary growth cohorts for sardine in Ganias and Lowerre-Barbieri (2018) exactly matched the 5 cohorts estimated in this study. For horse mackerel, Ganias et al. (2017) estimated up to 7 oocyte cohorts (using the fecundity ratio) which is again very close to present estimates ($5.8 \pm 0.8$). Mackerel exhibited a remarkably higher number of co-occurring cohorts compared to sardine and horse mackerel both in the present study ($n = 14.6 \pm 2.5$) and in Ganias and Lowerre-Barbieri (2018) ($n = 30$). The discrepancy between the present and modelled estimates for mackerel is apparently due to deficiencies of present methodology—i.e., clustering analysis and statistical decomposition of size frequency distributions—in distinguishing different cohorts amongst smaller oocyte classes. These cohorts of small oocytes present a high degree of overlapping, impeding their precise identification and classification. Indeed, the estimates of cohort specific fecundity (RFci) were quite higher for the smaller oocyte classes (early secondary growth), especially in mackerel, indicating that the predicted oocyte cohorts actually consisted of multiple cohorts. However, there was a consistency in the number of oocytes contained in cohorts in all the ovarian stages of all three species, indicating that the cohorts did not demonstrate significant deviances in the number of oocytes. Therefore, the output of the methods provides realistic results.

## Concluding remarks

An array of laboratory methods (histological and whole mount procedures) and statistical approaches (clustering analysis, modal analysis of oocyte distributions, and fecundity ratio) were presently used to evaluate previous assumptions on the link between the dynamic organization

of the ovary of multiple spawners and spawning dynamics. At the annual fecundity scale, it was shown that indeterminate spawners can display varying proportions of oocytes at the range between primary and secondary growth reflecting the degree of overlap between the period of secondary oocyte recruitment and the spawning period. Species like sardine with continuous oocyte recruitment throughout the spawning period exhibit high fraction of early secondary growth oocytes; this fraction gets lower in species like horse mackerel and mackerel that exhibit tapering or even complete cessation of secondary growth recruitment during the latter part of the spawning period. It was also shown that fishes like the Atlantic sardine with long spawning intervals and fast oocyte growth exhibit relatively few oocyte modes with clearly separated cohorts, while fishes like mackerel with shorter spawning interval and lower oocyte growth rate show increased number of coexisting oocyte cohorts. The present study covers the dynamics of secondary growth and primary growth oocytes; however, the recruitment from oogonia to primary growth oocytes needs to be further explored (Grier et al. 2009; Thomé et al. 2012; Wildner et al. 2013; dos Santos Schmidt et al. 2017). In addition, the thermal habitat, the latitudinal distribution, and other factors such as the availability of food and specificities in breeding behaviour should also be considered to obtain an overall view of fecundity regulation in fishes (Kjesbu and Witthames 2007; Ganias 2013; van Damme et al. 2014).

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## Declarations

**Conflict of interest** There is no conflict of interest to report.

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