The dynamics of postovulatory follicle degeneration and oocyte growth in Baltic sprat

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Abstract

Ovaries of Baltic sprat (*Sprattus sprattus balticus* S.) were analysed histologically to identify stages of postovulatory follicles (POF) and to assess the oocyte development pattern. Samples were taken every 3 h during a 24 h trawl survey conducted in the Bornholm Basin in April 2007. Gonad histology revealed spawning of sprat throughout the day which hampered the exact aging of POFs by the postovulatory follicle method. However, it was possible to define four stages of POFs, according to their histological features. The occurrence of these POF stages (I to IV) corresponded clearly to the development of the leading oocyte cohort. Further, the oocyte recruitment pattern revealed that the spawning batch can be identified prior to hydration. The POF stages I and II were present almost exclusively in vitellogenic ovaries, POF III were found in ovaries in the germinal vesicle stage, and POF stage IV was found in actively spawning fish with hydrated oocytes. Since only in very few ovaries POF were absent (5%), and in each ovary in general only one POF stage was present, the duration of POF degeneration approximately equals the average batch interval, i.e. the time lag between subsequent spawning events. The results of the present study will serve as basis for future studies on Baltic sprat oocyte recruitment and daily spawning fraction.

Keywords: oocyte recruitment; postovulatory follicles; *Sprattus sprattus*

Introduction

As many other small pelagic clupeoids, Baltic sprat release several batches of eggs over a protracted spawning season and show indeterminate oocyte recruitment (Alheit, 1988; Heidrich, 1925), i.e. the number of oocytes that will potentially be spawned during the breeding season is not determined prior to spawning. This means that individual potential fecundity cannot be estimated. However, the spawning stock biomass of these species can be determined from egg production methods, e.g. the Daily Egg Production Method, DEPM (Hunter, 1985) if information on a number of reproductive parameters is available. The use of DEPM requires the estimation of i) mean daily egg production from ichthyoplankton surveys and ii) the average daily individual fecundity. The latter involves the estimation of spawning frequency, batch fecundity, female weight and maturity status as well as sex ratio. Uncertainty in daily fecundity estimation of clupeids mainly resides in a lack of precision when estimating spawning frequency, either by the hydrated oocyte method or the postovulatory follicle (POF) method (Stratoudakis et al., 2006; Ganias, in press). The latter method requires histological preparation of ovaries to classify POF into distinct daily cohorts.

Although the Daily Egg Production Method is a valuable, fisheries independent tool to estimate the spawning stock biomass of small pelagic fishes, such as anchovy and sardines (Stratoudakis et al., 2006), it has never been applied to the Baltic sprat stock on a regular basis. Thereby, a DEPM application would be especially interesting for the Baltic sprat assessment, where conflicting results on stock size estimations from acoustic surveys and a multi species virtual population approach hampered quantitative studies on recruitment processes of sprat and cod so far, for example the estimation of predation pressure on cod eggs by sprat in the Bornholm Basin (Köster and Möllmann, 2000). The Bornholm Basin serves sprat and cod as an important spawning ground (Aro, 1989). Therefore, this area is surveyed since years by means of ichthyoplankton and fishery surveys and data needed to

implement the DEPM are available already (ICES 2002; Köster et al., 2005; Haslob et al., 2011). Thus, besides a future application even a retrospective stock assessment with the DEPM in order to validate former assessments is possible. However, the lack of adequate estimations of spawning frequency hampered the implementation of the DEPM for this pelagic species in former studies (Kraus and Köster, 2004). Neither have detailed histological investigations of Baltic sprat ovaries been published before nor was it attempted to stage or age POFs of sprat.

The best way to estimate the duration of POF is by laboratory tank experiments, where fish spawn under controlled conditions and adult fish are sampled at a specified time after spawning (Macewicz et al., 1996). As this is often not practicable, an alternative method was developed to estimate the spawning fraction from a series of samples collected over a 24 h period in the field (Alheit et al., 1984; Goldberg et al., 1984). On the basis of such samples distinct histological stages of POF have to be defined in relation to the daily peak spawning time. Therefore, this method is strictly speaking only suitable for fish species with a daily synchronous spawning behaviour, e.g. the Northern Anchovy (*Engraulis mordax*; Hunter et al., 1985) or the Mediterranean Sardine (*Sardina pilchardus sardina;* Ganias et al., 2003). For sprat, contradictory observations on the diel spawning behaviour are reported in literature. Simpson (1971) reported the diel spawning of Sprat from the Irish Sea between 22:00 p.m. and 06:00 a.m., with a peak between midnight and 04:00 a.m. For Baltic sprat, an asynchronous diel spawning pattern was observed (Alekseev and Alekseeva, 2005).

In the present paper, we introduce a method for staging POF according to (i) their histological features of degeneration, (ii) the dynamics of the oocyte growth of the most advanced oocyte cohort. This study is the first to analyse the histological features of ovarian maturation stages and POFs of sprat in detail.

Material and methods

Baltic sprat were sampled with a pelagic trawl during a research cruise with RV "ALKOR" in the Bornholm Basin during the main spawning season in April 2007 (Fig. 1). Pelagic trawls were carried out in the central basin defined by the 80 m depth line every 3 h over a 24 h period (n=9). From each haul a 2 kg sub-sample (n≈200) of sprat was preserved in a buffered 10% formaldehyde solution. To assure proper fixation of the samples, the body cavity of each fish was slit open prior to fixation.

Histological processing

In the laboratory, at least 20 females were taken randomly from each sample for histological analysis of the ovaries. From these specimens, the total body weight (g), ovary free body weight (g), and total length (nearest 0.1 cm) were measured. The ovaries were removed, weighed to the nearest 0.001 g, and fixed again in a 4%-formaldehyde solution. A tissue sub-sample from the middle part of each ovary was embedded in paraffin. Histological sections of 3 µm were cut and stained, using the Hematoxilin-Eosin method. All histological sections of ovaries were analysed for the developmental stage of oocytes and the presence of postovulatory follicles (POF). Five oocyte developmental stages were distinguished (Brown-Peterson et al., 2010): primary growth, cortical alveoli, vitellogenesis, germinal vesicle migration (GVM) and hydrated oocytes (Fig. 2) forming the basis to classify the sampled females into three distinct phases of the reproductive cycle (Brown-Peterson et al., 2010): i) developing, ovaries containing only vitellogenic oocytes, ii) spawning capable, ovaries with oocytes undergoing GVM, and iii) actively spawning, hydration is present in the ovary.

According to their histological features, POFs have been assigned into different stages of degeneration (Fig. 3; Tab. 1). Referring to observations from other clupeid fishes, the shape,

the size of lumen and the state of the granulosa layer were mainly used for that purpose (Ganias, 2003; Hunter and Macewicz, 1985).

Oocyte recruitment in relation to POF stages

To assess the oocyte development pattern, 40 ovaries from two consecutive hauls (haul 6 and 7) were processed with the whole-mount method as described below. In addition, tissue of the same ovaries was analysed histologically. Thereby it was possible to relate the oocyte developmental pattern directly to histological results. For the whole mount method at least 50 mg of ovary tissue was removed from the sample. The oocytes were then separated into different size classes with 600 µm, 300 µm and 150 µm sieves. After separation oocytes were photographed, automatically measured to the nearest µm (diameter) and counted with image analysis software following the procedures described in Dominguez-Petit (2007). The separation with sieves into different size groups facilitates the image analysis, as no large oocytes will cover smaller ones. All particles were measured and counted and finally pooled and analyzed together. Thus, no bias in size frequency was introduced by sieving the material. However, for subsequent analysis only oocytes >200 µm diameter were taken into account in order to exclude primary growth oocytes (Ganias et al., 2003). The oocyte diameters were grouped in 25µm intervals and length frequency distributions were established for each analysed ovary. Cohorts were identified in each oocyte size frequency distribution and for each cohort the median oocyte diameter was calculated. In most cases there was a clear gap between the two cohorts. In the few cases with overlapping cohorts, the value with the lowest frequency between the two distribution modes was defined as lower and upper limit, respectively. The median was chosen as some frequency distributions of the more advanced oocyte cohorts showed significant deviations from normal distribution (K-S-Test; $p<0.05$). By sorting the analysed ovaries according to their median oocyte diameter of the leading cohort,

the oocyte growth dynamics was analysed with respect to the according POF stage and ovarian maturation stage of each analysed ovary. In order to estimate the relative duration of POF stages, those ovaries for which oocyte diameters were measured and which contained POFs were sorted by the median diameter of the most advanced oocyte cohort to display the whole spawning interval. Further, the POF stage and gonadal development stage were assigned to each ovary.

Results

The histological classification of all analysed ovaries revealed 23% (±15% SD) females with hydrated oocytes, 41% (±19% SD) in the vitellogenic and 36% (±20% SD) in the germinal vesicle migration stage (Tab. 2). Spawning activity showed an asynchronous diel pattern, as females with hydrated ovaries were found throughout the day. However, the proportion of females with hydrated ovaries varied largely between hauls over the entire 24 h period. The highest proportion of hydrated females was found at 06:00 p.m. with nearly 50% of females containing ovaries with hydrated oocytes. The lowest value was found at noon with no females having hydrated oocytes (Fig. 4a). In 95% of analysed ovaries POFs were detected. Also the occurrence of different POF stages showed an asynchronous pattern throughout the day (Tab. 2). The variability of POF stage proportions between the sub-samples was high for every POF stage. The highest range was detected for POF I with 0% at 06:00 a.m. and 65% at noon (Fig. 4b).

The analysis of oocyte size frequency revealed the presence of two clearly distinct cohorts of secondary growth stage oocytes: from the pool of early vitellogenic oocytes, subsequent cohorts develop one by one with increasing oocyte diameter until spawning.

The median oocyte diameter in ovaries with only vitellogenic oocytes ranged from 410 µm to 558 µm, the median of oocytes in the germinal vesicle migration stage ranged from 568 µm to 813 µm (Fig. 5), and the hydrated oocytes ranged from 877 µm to 1388 µm in diameter. While the size increase was clearly visible for the leading cohort, the median diameter of the second cohort remained constant around 400 µm. Although the median values between two cohorts become distinct in early development (Fig. 5), there was an overlap in oocyte size distribution during early vitellogenesis becoming completely separated when the leading cohort develops towards the germinal vesicle migration stage.

The occurrence of POF stages (I to IV) corresponded clearly to the development of the leading oocyte cohort. The POF stages I and II were present almost exclusively in vitellogenic ovaries, and similarly POF III were found in spawning capable females (GVM). POF stage IV was found in actively spawning fish with hydrated oocytes (Fig. 5). Remains of the latest POF stage could also be detected in parallel with most recent POFs in early vitellogenic ovaries. Considering that in very few ovaries of the analysed material POF were absent (5%), and in each ovary in general only one POF stage was present, the duration of POF degeneration approximately equals the average batch interval, i.e. the time lag between subsequent spawning events.

Discussion

The investigated oocyte development pattern of sprat shows a distinct hiatus between oocytes in the germinal vesicle migratory stage prior to hydration and the remaining reserve oocytes. A similar pattern has been observed for the anglerfish *Lophius litulon* (Yoneda et al., 2001), for *Siganus canaliculatus* (Hoque et al., 1999) and, as an example for a clupeid fish, Mediterranean sardine *Sardina pilchardus sardina* (Ganias et al., 2004). For the latter species

it was possible to estimate batch fecundity by using not fully hydrated females. Our results demonstrate also for Baltic sprat that it is possible to use females in the germinal vesicle migratory stage to estimate batch fecundity. This will be of advantage for the sampling procedure as it is sometimes difficult to obtain sufficient numbers of hydrated females from one haul, since their proportion was shown to be highly variable. Moreover, the risk of including partly ovulated ovaries of already running ripe females into the batch fecundity analysis would be minimized. Thus, checking histologically for recent POFs is not necessary as it is recommended when using fully hydrated individuals. The auto-diametric method to estimate fecundity (Thorsen and Kjesbu, 2001) can be easily applied. Hence, increasing number of samples may enhance fecundity estimation accuracy. However, the precision of the auto-diametric method should be tested statistically prior to a possible application to Baltic sprat. Witthames *et al*. (2009) reported that this method was less accurate when applied to the indeterminate spawner hake (*Merluccius merluccius*), compared to species with determined oocyte recruitment as cod (*Gadus morhua*) or plaice (*Pleuronectes platessa*).

Unlike other clupeid fish species, there is some evidence that Baltic sprat may not show a clear diel synchronous spawning behaviour (Alekseev and Alekseeva, 2005), a prerequisite to estimate spawning frequency by means of the postovulatory follicle method (Ganias, in press). However, the diel spawning behaviour of sprat remains controversial as also the opposite has been reported for sprat. Simpson (1971) was able to detect diel peak spawning of sprat around 2:00 a.m. in the Irish Sea from ichthyoplankton samples by counting the earliest cleavage egg stages. Vertically resolved ichthyoplankton samplings over more than one 24 h sampling conducted by Balzar (1994) revealed Baltic sprat spawning at night around 03:00 a.m. near the surface. However, this study was restricted to one sampling period in July, *i.e*. at the end of sprat spawning season. It has been reported before that sprat spawning shifts from below the halocline at the beginning of the spawning period to surface water layers towards

the end of the spawning season in the Baltic basins (Alekseev and Alekseeva, 2005). This shift in spawning depth may also affect the diel spawning behaviour which therefore may change over the course of the prolonged spawning season. Findings of the present study confirmed the findings by Alekseev and Alekseeva (2005), since females with hydrated oocytes and recent POF were found throughout the day. Although there was a concentration of hydrated females found around 06:00 p.m. it is doubtful that this result verifies a synchronised diel peak spawning, because (i) the occurrence of recent POF does not confirm this pattern, as no increase in proportion of recent POFs occurs during the late evening/early morning hours after this peak, and (ii) if peak spawning occurred in the afternoon and at dusk, the elevated proportion of hydrated females should be found prior to the peak spawning for the duration of the hydrated stage. For the observed pattern that would translate into approximately 6 h duration for the hydrated stage from noon until the observed peak at 06:00 p.m.. Given the mean percentage of 23% hydrated females during the sampling this would result in more than one spawning per day and female, which seems quite unrealistic.

The lack of diel spawning synchronicity is in contrast to many other clupeid species, e.g. for *Sardina* spp. or *Sardinops* spp. (Ganias et al. 2003; Macewicz et al. 1996), which synchronise their spawning during few hours at night time. For these species a reference time exists for the estimation of POF stages by means of field samplings. Alday et al. (2008) used an alternative approach to determine the POF duration in anchovy (*Engraulis encrasicolus*). They first defined different stages of POF solely based on histological features of degeneration, and then aged these defined stages in a second step by using information from tank experiments. Since no information from tank experiments with sprat was available for this study and no clear spawning peak during the day was found to serve as reference time, the exact determination of POF duration was not possible in the present study. Nevertheless, it was possible to identify four different stages of POF with respect to their histological features and their state

of deterioration and to relate these stages to distinct maturation stages. Nearly all analysed ovaries contained POF (95%) and in each ovary in general only one POF stage was present. Only in ovaries with very recent POFs some remains of the latest POF stage could be detected. Thus, it can be concluded that the duration of complete POF resorption virtually equals the average batch or spawning interval in Baltic sprat. In very few individuals no POF were detected. These females probably formed their first batch in the early spawning season in April.

Similar to the case of diel spawning behaviour, reports on the spawning interval of sprat remains controversial. Historical observations by Heidrich (1925) reported a spawning interval of 7 to 9 days from the Kiel Bight area but it was criticized that Heidrich might have underestimated the spawning frequency, because of methodical shortcomings (Alekseev and Alekseeva, 2005; Kraus and Köster, 2004). In his study the number of developing oocytes was divided by the number of hydrated oocytes which is not correct in species with indeterminate oocyte recruitment. The most recent estimate is provided by Alekseev and Alekseeva (2005) who reported a spawning interval of 4.3 days for sprat from the south eastern Baltic area. Approximately four days on average was reported for sprat from the Bornholm Basin (Kraus and Köster, 2004). However, all these estimations are solely based on the use of the hydrated oocyte stage as daily spawning marker. This approach is problematic because hydrated females may be oversampled due to spawning aggregations or higher vulnerability to the sampling gear (Alheit, 1984; Stauffer and Piquelle, 1985). Kraus and Köster (2004) found exceptional high spawning fraction values of up to 63%-93% for sprat from the Bornholm Basin in April. They explained these extreme values by ongoing migration of ready to spawn females into the spawning area at the beginning of the spawning season, but it could also be due to the forming of spawning aggregations which will result in oversampling (Ganias, in press).

Duration of less than 24 h for the first POF stage was reported for several clupeid species such as *Sardina pilchardus pilchardus* off Portugal (ICES, 2000), *Sardinops melanostictus* off Japan (Aoki and Murayama, 1993), and *Sardinops sagax musica* off Chile (Claramunt and Herrera, 1994). In contrast to sprat, for these species only three POF stages have been described. The complete POF duration of northern anchovy lasts longer than 72 h (Hunter and Macewicz, 1985). For the Mediterranean sardine (*Sardina pilchardus sardina*), the first POF stage was reported to last 10 h (Ganias *et al*., 2003), a comparably shorter duration than for most other clupeid species. These differences might be related to different temperatures prevailing in the investigated areas, as the resorption of POF is known to be strongly dependent on the ambient temperature (Fitzhugh and Hettler, 1995), and also the growth rates of oocytes have been reported to increase with increasing temperatures (Kurita *et al.*, 2011). Therefore, the POF stage duration and the duration of ovarian developmental stages of Baltic sprat might vary over the prolonged spawning season due to an increase in ambient temperatures over the spawning season lasting approximately from March to June. However, this issue should be addressed in future investigations to further enhance the understanding of POF degeneration and thus spawning frequency of Baltic sprat.

From the oocyte size distribution it can be seen that right after releasing the spawning batch the next cohort of oocytes begins to increase in size to form a new batch. For Mediterranean sardine a spawning interval of 10 to 11 days was reported (Ganias *et al*., 2003), an interval considerably longer than reported before for other stocks, e.g. maximum values of up to 6 days were reported for sardines off the Atlantic coast off Spain (ICES, 2009). This was explained by the oligotrophic characteristic of the Mediterranean Sea, as the egg production and spawning frequency might be influenced by food availability and thus condition of the fish. Peak spawning of sprat is synchronised with the peak production period of calanoid copepods in the central Baltic, the major prey items for sprat (Möllmann *et al*., 2004; Möllmann *et al*., 2003). Thus, feeding conditions for Baltic sprat are favourable during the

peak spawning period. Petrova (1960) reported an accelerated oocyte development of sprat which was explained by optimum food availability and also favourable temperature conditions. Results of the present study revealed that in Baltic sprat the most degenerated POFs and hydrated oocytes co-occur together in the same ovary. This could be explained by a comparatively slow POF degeneration, due to low ambient water temperatures, or a relatively fast oocyte development, due to favourable feeding conditions, or a combination of both effects. The POF degeneration as well as oocyte growth may depend on environmental conditions. Thus, the association of the respective POF stages to oocyte recruitment might vary seasonally. It was not possible to investigate the relative effect of temperature or prey availability on POF duration and oocyte recruitment in the present study, as only samples from one cruise were analysed.

The present study has to be seen as a pilot study since sample sizes were limited and only one single 24 h period for one time point of the year could be realised. More effort in this direction would help to clarify the picture. Several 24 h fishery samplings should be carried out to get a more reliable result on the diel spawning pattern. Vertically resolved ichthyoplankton samplings over more than one 24 h sampling are required to elucidate the spawning behaviour of Baltic sprat. However, the results of the present study provide the basis for future studies on sprat spawning dynamics, in order to compare reproductive parameters between different stocks, years and seasons. So far all estimates of sprat spawning frequency are based on assumptions about the duration of used spawning markers and not on direct observations or experimental results. Therefore, it is strongly recommended to further investigate this important parameter in sprat.

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Tab. 1: Histological features used to identify different stages of postovulatory follicles (POF).

	Histological features
POF I	The POF has a clear convoluted shape and a rather big lumen. It consists of two cell layers, theca and granulosa with clearly visible nuclei and relatively thin width with similar morphology to the follicle wall in advanced vitellogenic oocytes. The cross sectional area is larger compared to that of other identified POF stages. This type of POF occurred exclusively in ovaries where vitellogenesis was the most advance stage of oocyte development (Fig. 3a).
POF II	The POF has still a distinct convoluted shape but the lumen has become smaller. The two cell layers are still distinguishable but first signs of degeneration are visible: Vacuoles become visible and nuclear pycnosis occurs. The thickness of the two cell layers has clearly increased and granulosa cells are more rectangular. Again, this type of POF occurred exclusively in ovaries where oocytes have not developed further than vitellogenic stage (Fig. 3b).
POF III	The convoluted shape begins to disappear, as the follicle is being resorbed. The POF lumen shrinks and is no longer clearly distinct. It is not possible to distinguish between the two cell layers. The cross sectional area has clearly decreased compared to the two first POF stages. The thickness of the two cell layer has decreased again due to degeneration. This POF type occurred in ovaries with early and advanced germinal vesicle migration stage or in ovaries with early hydrated oocytes (Fig. 3c).
POF IV	The convoluted shape and lumen are not visible anymore. The follicle has shrunk considerably, becoming more compact and the cross sectional area is very small compared to the other stages. This POF type occurred in ovaries with advanced germinal vesicle migration and hydrated oocytes (Fig. 3d). Remains of this stage are also detectable in vitellogenic ovaries with very recent POFs.

Tab. 2: Date and time of sampling, n = number of sub-sampled females for histologic analysis; sex ratio as proportion of females from at least 200 specimens; fraction of maturity stages and POF stages derived from histological analysis.

haul	date	time	n	sex ratio	Fraction of:							
					vitellogenesis	GVM	hydrated	POF I	POF II	POF III	POF IV	No POF
1	16 April 2007	06:18	21	0.41	0.19	0.57	0.24	0.00	0.10	0.38	0.43	0.10
2	16 April 2007	09:00	23	0.48	0.30	0.57	0.13	0.09	0.17	0.35	0.30	0.09
3	16 April 2007	12:00	23	0.22	0.86	0.14	0.00	0.65	0.22	0.09	0.00	0.04
4	16 April 2007	15:00	20	0.22	0.50	0.10	0.40	0.25	0.25	0.05	0.40	0.05
5	16 April 2007	18:00	20	0.31	0.35	0.15	0.50	0.20	0.10	0.15	0.55	0.00
6	16 April 2007	21:00	20	0.58	0.50	0.25	0.25	0.10	0.25	0.35	0.20	0.10
7	17 April 2007	00:00	21	0.54	0.38	0.38	0.24	0.14	0.19	0.38	0.24	0.05
8	17 April 2007	03:00	21	0.30	0.38	0.48	0.14	0.29	0.10	0.38	0.19	0.05
9	17 April 2007	06:00	21	na	0.24	0.57	0.19	0.10	0.10	0.52	0.29	0.00

Table and Figure captions

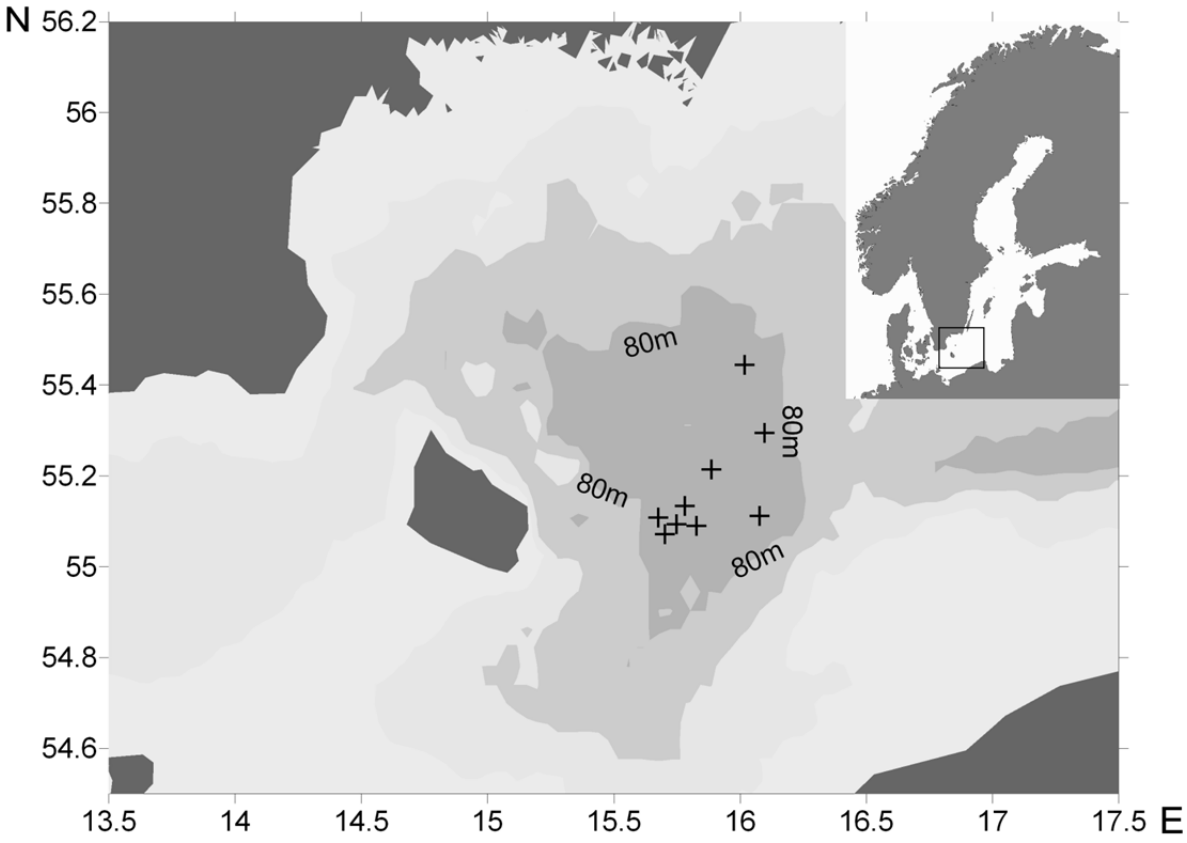

Fig. 1: Map of the sampling area in the Bornholm Basin (ICES Sub-Division 25). Fishery hauls 1 – 9 (crosses) were conducted within the 80 m isobath.

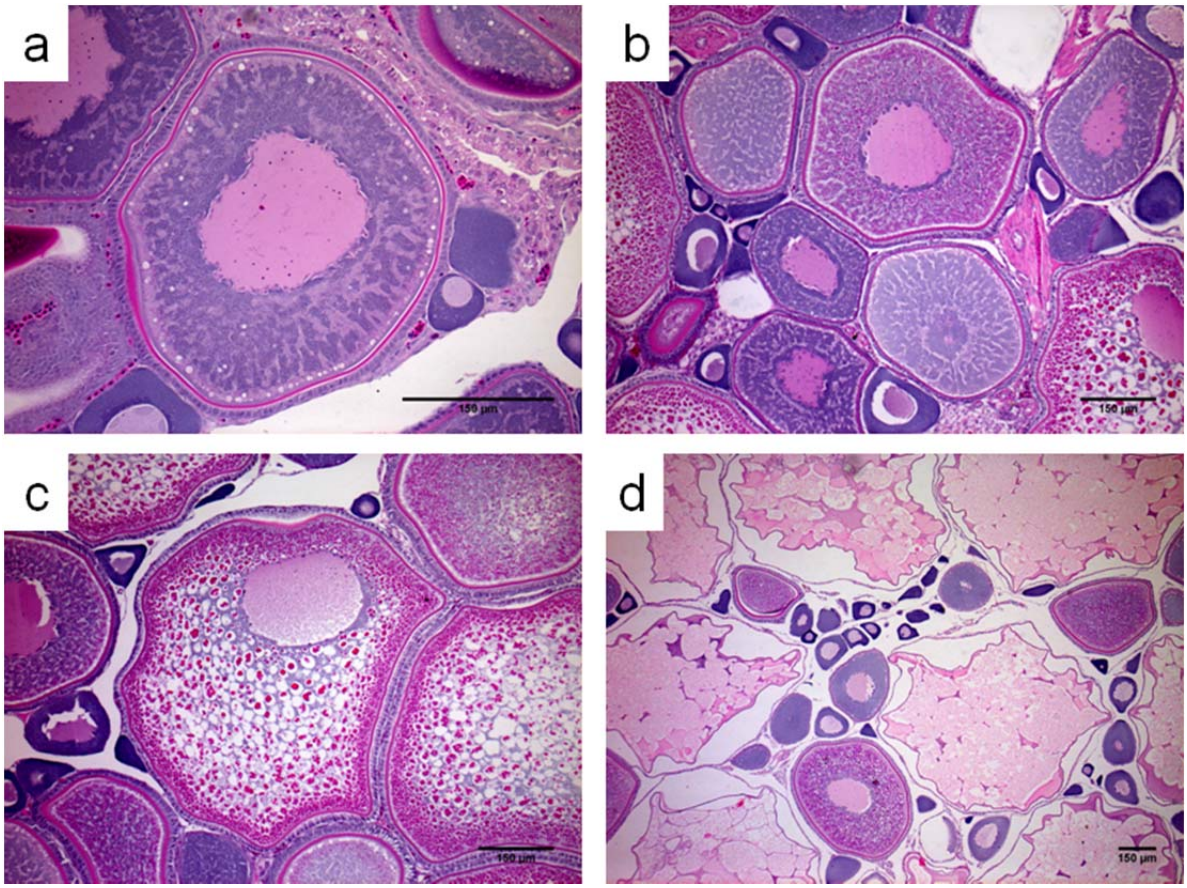

Fig. 2: Stages of oocyte development. (a) cortical alveoli, (b) vitellogenesis, (c) germinal vesicle migration and (d) hydrated.

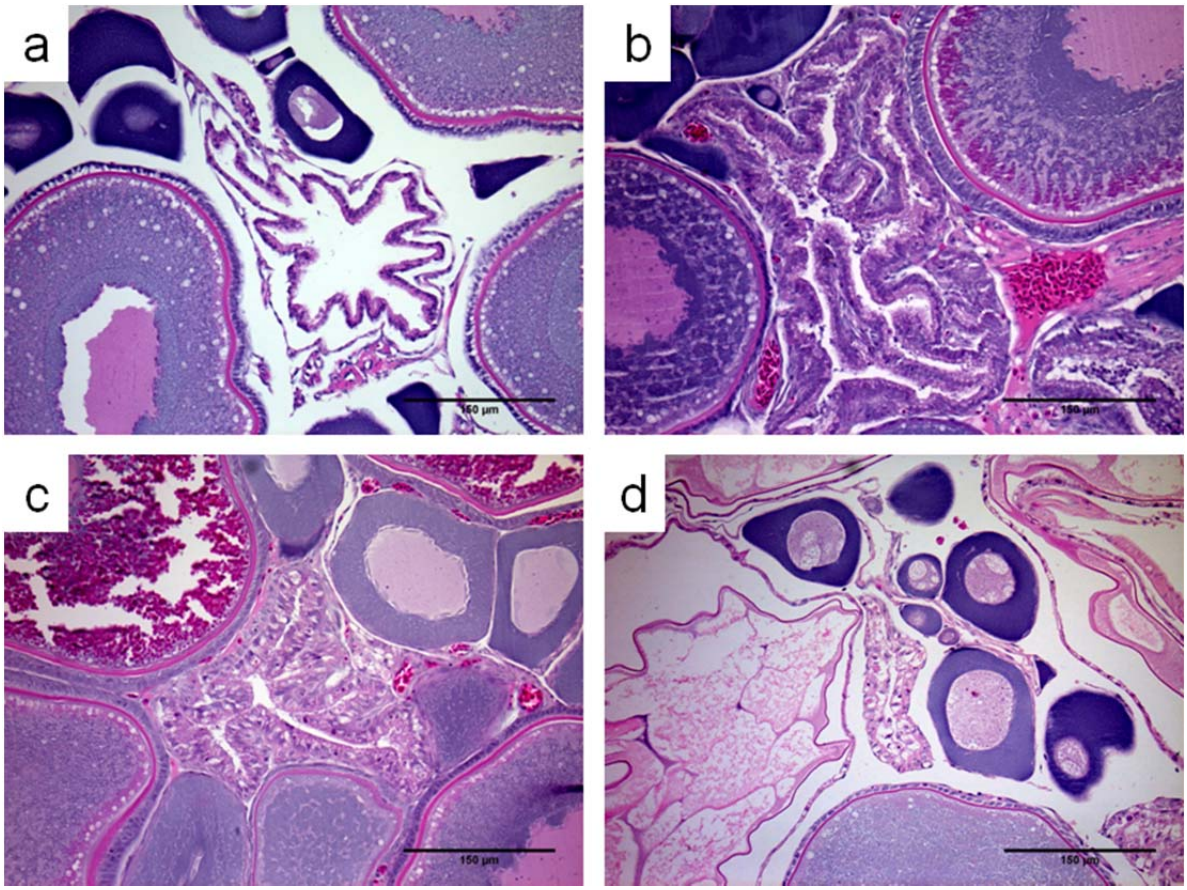

Fig. 3: Stages of postovulatory follicles of Baltic sprat. (a) POF I, (b) POF II, (c) POF III and (d) POF IV.

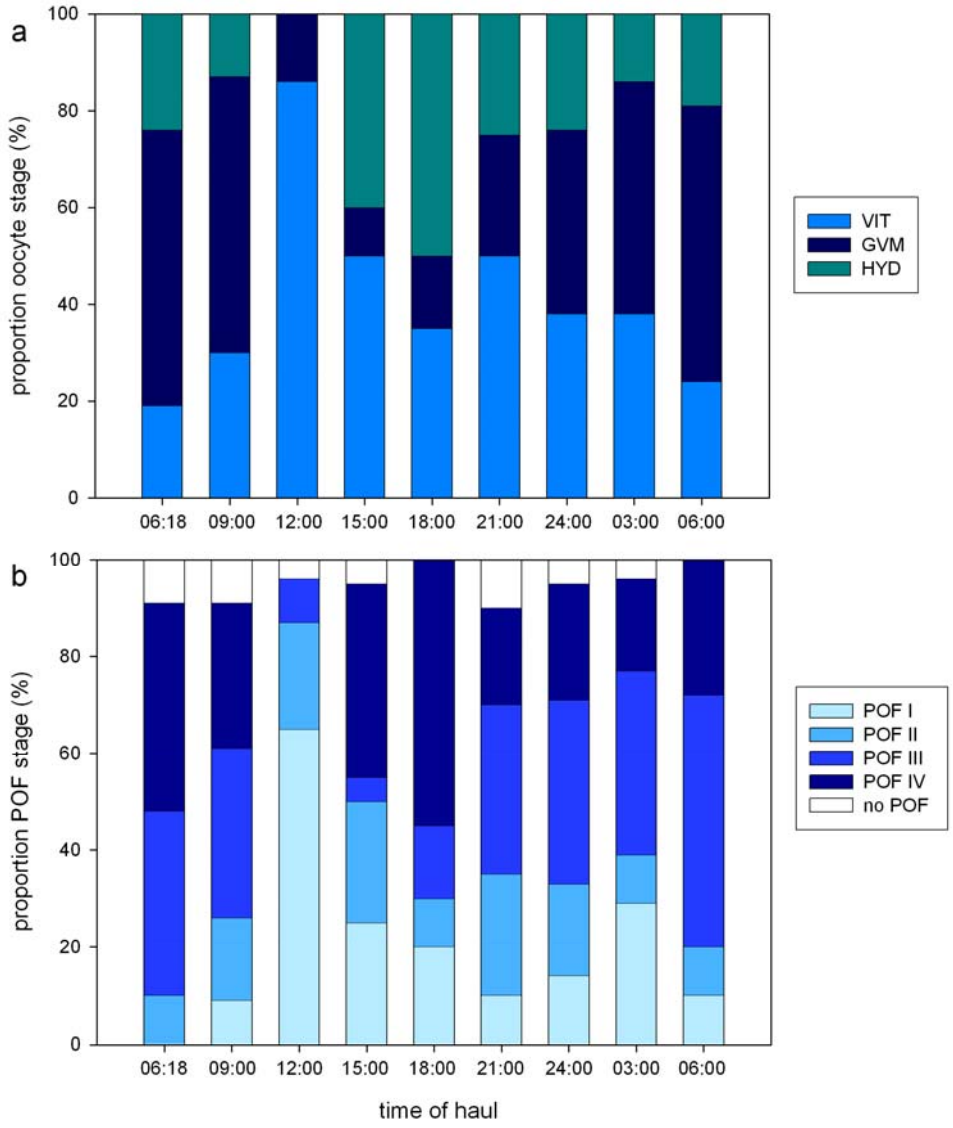

Fig. 4: Share of different oocyte stages (a) and POF stages (b) in fishery hauls conducted over the 24 h period.

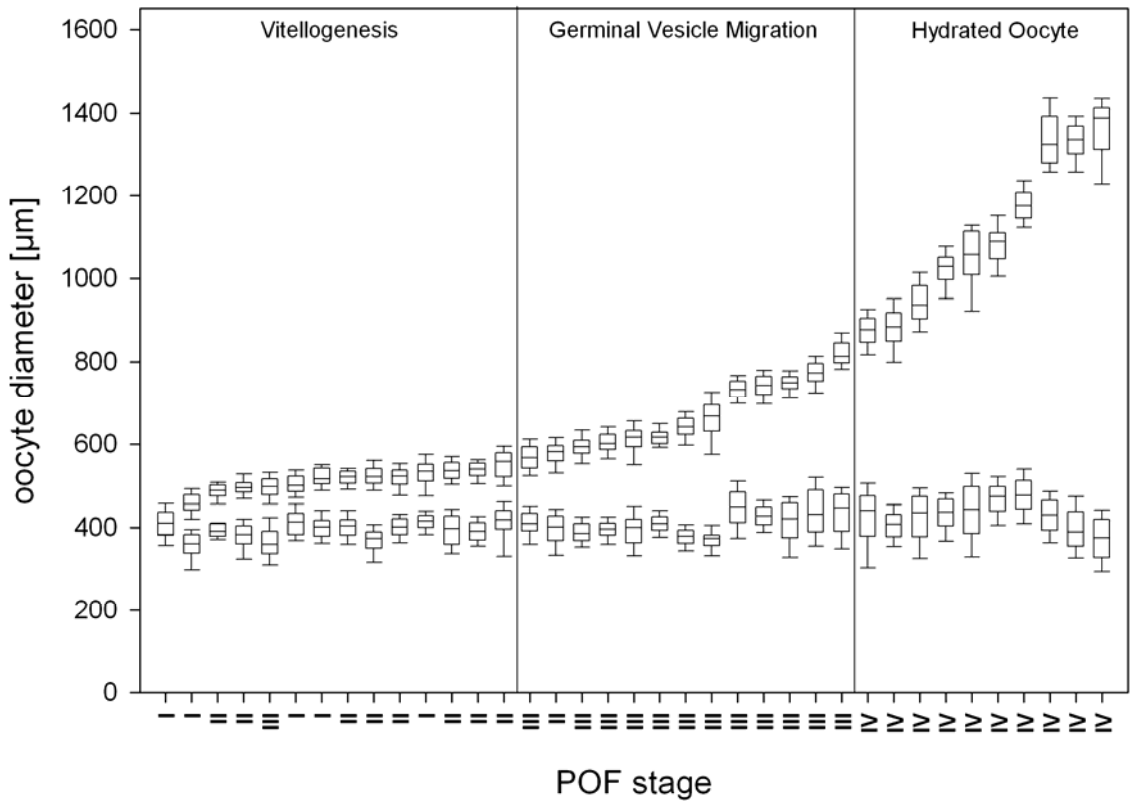

Fig. 5: Development of median diameters of the reserve stock of oocytes (lower row) and developing oocytes (upper row) with corresponding POF stages. Box displays 25th and 75th percentiles, vertical lines display 10th and 90th percentiles.