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Histological analysis of samples from 2019 and 2020

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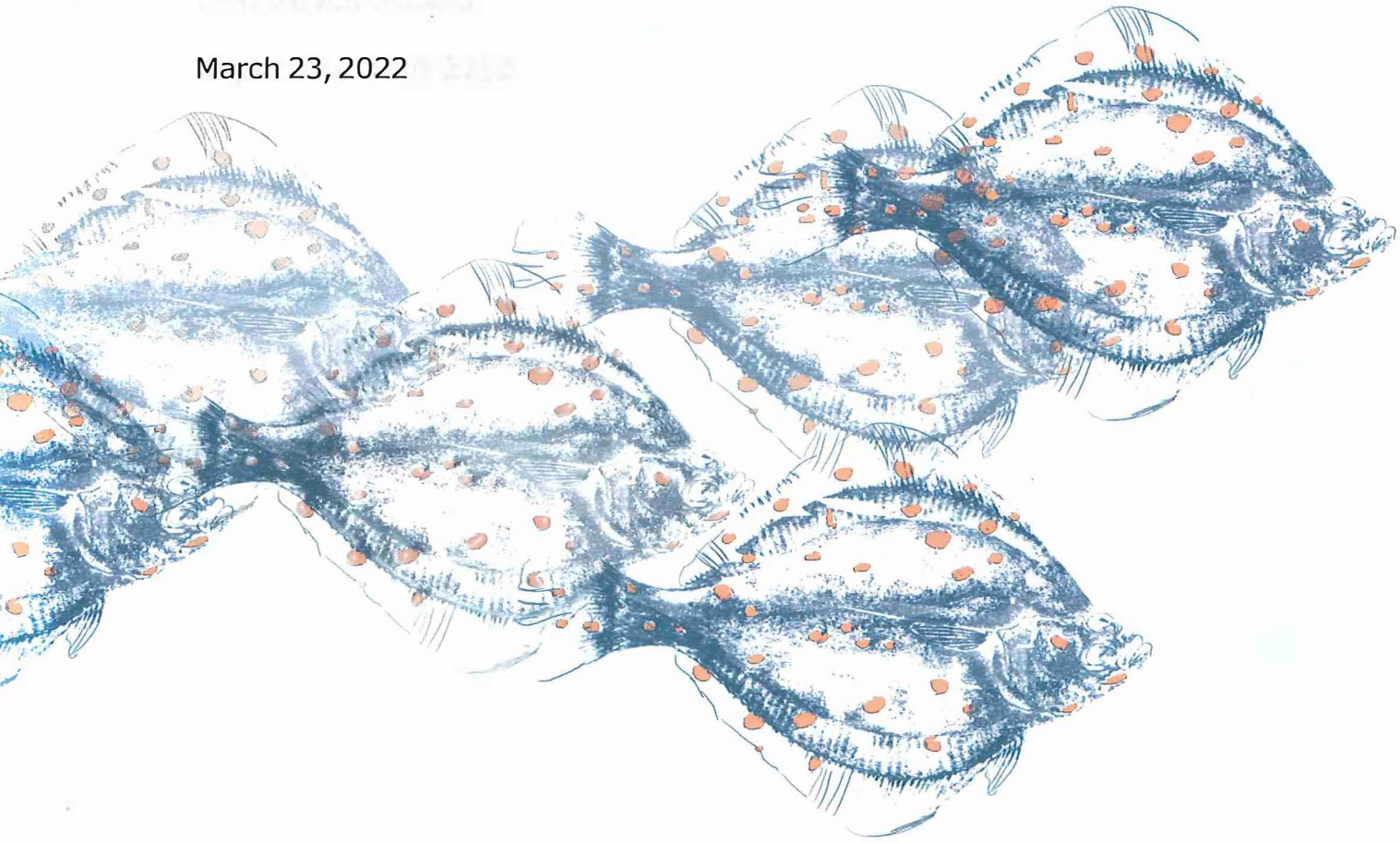
CVO Report 22.001

March 23, 2022



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CVO report: 22.001

Commissioned by:
Ministerie van LNV
Directie VLG
drs. H.H.A. van Bemmel
Postbus 20401
2500 EK Den Haag

Project number: 4311300073
BAS code: KB-36-002-011

Publication date: March 23, 2022

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This research is conducted as part of the knowledge base connected to the statutory task programme "fisheries research" and subsidised by the Dutch Ministry of Agriculture, Nature and Food Quality.

DOI: https://doi.org/10.18174/562442

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VAT nr. NL 8089.32.184.B01

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Summary

In recent years aberrant maturity of sole (*Solea solea*) was recorded in WMR market samples, and during the North Sea beam trawl survey in August 2018 spawning female plaice (*Pleuronectes platessa*) was caught. These plaice were exceptionally skinny. Although the recordings could have been treated as incidents, it was decided to further examine these signals, and investigate if the maturation cycles in sole and/or plaice are changing. It was proposed to further look into the gonad development and spawning cycle of plaice and sole in the North Sea. As maturity can only be reliably studied macroscopically in the period directly prior to spawning and during spawning (ICES, 2018), microscopic maturity staging was used to reliably do year-round observations.

The research question of this study is twofold, first focussing on the development of flatfish maturity throughout the year based on histological samples, and then checking if the observed development aligns with the expected development.

Samples from fish were taken monthly during the regular market sampling, from April 2019 till March 2020. The fish were sampled from both the northern and the southern part of the North Sea. For plaice the border was set at 53°N and for sole at 52°N to separate the spawning components. For female fish statistical analyses were conducted to model the maturity over the months, correcting for some other covariates, using oocyte and egg proportion and diameter as the responses. No statistical models were run on the male specimen, as only the screening for presence or absence of sperm cell development stages was carried out.

The development of cell types, especially the previtellogenic stage towards the vitellogenic stages is difficult to model. Thus it is hard to statistically underpin the maturation over time. This is largely due to the low number of data. The oocyte diameter increases during the maturation and following spawning cycle, from a small diameter after the previous spawning season, towards larger diameters as the nest spawning season comes closer. The diameters can reliably be modelled over time, for both plaice as well as sole.

The expected development for both species is that the relative number of vitellogenic oocytes and their diameters increase towards the spawning season, and decline soon afterwards. As a consequence, the relative number of previtellogenic oocytes is expected to increase soon after the end of the spawning season, when the maturation cycle starts again.

The plaice development seems to be aberrant from the expectation, especially in the southern North Sea. Plaice, as a capital winter spawner, is to be expected to build up the number of oocytes and let them evolve gradually towards the spawning season. The relatively high proportion of vitellogenic oocytes found from June till August, followed by the decline and an increase towards December is not in line with the expectation. The decline of the relative number of vitellogenic oocytes is most likely due to spawning activity, as post-ovulatory follicles were encountered in the samples from September to November 2019. Incidental spawning activity in summer for plaice in the southern North Sea is in line with the signals from the North Sea beam trawl survey.

For sole no changes in the maturity pattern were found in this study, despite the signals from market sampling that sole gonads seemed to develop earlier in the season towards spawning than expected. The number of fish analysed in this study are low, due to the time-intensive labour of the histological analyses. Possibly the low number of samples missed any aberrant development in sole. However, analyses of gonadosomatic index and condition index (Fulton's K) development over the year in the time-

series from 1996 to 2019 did not show any changes over time (Chen *et al*. 2019, Wilkes 2020). This supports the results of the current study that no changes in gonad maturation are found.

1 Introduction

Timing of spawning of plaice (*Pleuronectes platessa*) is generally from December till February, and for sole (*Solea solea*) March-June. In recent years aberrant maturity of sole was recorded in WMR samples from the auction to study biological parameters such as age and maturity. During the North Sea beam trawl survey in August 2018 spawning female plaice was caught. These plaice were exceptionally skinny. Although the recordings could have been treated as incidents, it was decided to further examine these signals, and investigate if the maturation cycles in sole and/or plaice are changing. Gonad development and spawning cycle of plaice and sole in the North Sea were studied. As maturity can only be reliably studied macroscopically in the period directly prior to spawning and during spawning (ICES, 2018), microscopic maturity staging was used to reliably do year-round observations.

Plaice is considered to be a capital, determinate batch spawner. Capital spawners acquire the energy needed for reproduction prior to the spawning period (van Damme, 2013). As a consequence, the spawning success is quite independent of the food availability in the spawning season. The number of oocytes that can develop into eggs is set at the start of the spawning season (determinate), and spawning occurs in batches throughout the season (batch) (van Damme, 2009). The main spawning period in the North Sea is between December and March, with peak spawning in January-February.

The northern part of the sole stock in the North Sea, on the other hand, is an indeterminate spawner, which means that the species can spend energy in development of oocytes and sperm cells throughout the year. The energy intake shortly before and during the spawning season may affect the reproductive success. Gonadal development in sole in the southern North Sea is assumed to be more in line with development in plaice, starting well before the spawning season. The question also arises if sole in the southern North Sea are switching to an indeterminate spawning type. The spawning period of Sole in the North Sea occurs late winter and spring, with earlier spawning in the southern North Sea and later spawning of the northern sole stock (Fincham *et al*. 2013).

The research question of this study is twofold:
(a) how does the maturity of flatfish develop throughout the year, based on histological samples?
(b) is the observed development is in line with the expected development?

For the study, new samples were collected from April 2019 till March 2020, and the results were compared with data from previous studies.

2 Materials and methods

2.1 Biological

2.1.1 Samples

Samples from fish were taken monthly during the regular market sampling, from April 2019 till March 2020 (Table 2.1). The sampling was spread over the different auctions, to spatially cover the targeted stocks as good as possible. Histological slides were prepared following the manual (van Damme, 2022). As the number of fish sampled from the market sampling was large, and preparing and analysing histological slides is time-consuming, a selection of fish was made (Table 2.2). First time spawners were excluded from the selection, and it was made sure that the total dataset covered all sampling months.

Table 2.1. *Number of gonads collected from the WMR market sampling*

	Pleuronectes platessa		***Solea solea***	
	female	male	female	male
2019 (May-December)	3400	424	2806	388
2020 (January-April)	1176	179	533	67
total	4576	603	3339	455

Table 2.2. *Number of gonads selected for histological analysis*

	Pleuronectes platessa		***Solea solea***	
	female	male	female	male
2019 (May-December)	49	23	36	19
2020 (January-April)	25	9	18	9
total	74	32	54	28

2.1.2 Histological maturity staging

Males

For males, maturity was assessed through the presence of sperm cells at the different development stages (Table 2.3). Staging followed procedures from Blom (2022).

Table 2.3. *Sperm cell development stages as used for the proportion variables (male fish).*

Term	**Acronym in figures**	**Explanation**
Spermatogonia	S.togonia	Primary Spermatogonia (Sg1) are oval shaped cells, 7-22µm in diameter that appear isolated or in small groups. Both nucleus and cytoplasm of Sg1 are slightly basophilic. The nucleus frequently occupies 2/3 of the cell surface and may have a conspicuous strongly basophilic nucleus at the centre (Dark blue). Sg1 are frequently accompanied by smaller Sertoli cells (4-7 µm) in diameter. Secondary spermatogonia (Sg2) are polygonal cells, 4-7 µm in diameter that appear densely packed in nests of 8-35 cells. The nucleus has intermediate basophily (deep purple) and occupies

Term	Acronym in figures	Explanation
		nearly the entire cell. Cytoplasm is acidophilic and very reduced. No nucleoli are visible.
Spermatocytes	S.tocytes	Primary spermatocytes (Sc1) are polygonal to round cells 4-8 µm in diameter that appear more loosely clustered (frequently over 50 cells) and in larger numbers than Sg2. The nucleus of Sc1 is circular and strongly basophilic (dark blue) with no nucleolus visible. The cytoplasm has low basophily (light purple and unlike Sg2 is clearly distinguishable under light microscopy. Secondary spermatocytes (Sc2) are similar in appearance and numbers to Sc1 but smaller 3-6 µm, more basophilic (both nucleus and cytoplasm) and more densely packed. Sc2 are relatively brief development stage that are abundant only on 30% of the spawning capable individuals.
Spermatids	S.tids	Spermatids are markedly smaller than the previous spermatogonic stages, their nucleus remaining strongly basophilic and the cytoplasm has intermediate basophily. Their overall size (2-4 µm) is low compared with Sc2. They appear in very dense nests that may contain several hundred cells.
Spermatozoa	S.tozoa	Spermatozoa first appear with their heads and tails aligned within recently burst spermatid cysts in "parachute" form. Their heads are tiny (1-3 µm) and strongly basophilic (dark blue), their tails being long and mildly basophilic (light purple). During spawning they are released to the lumen of the lobules where they form dense aggregations.
Spermatozoa in spermatoduct	S.tozoa in S.toduct	Latest stage in the development; when spermatozoa are present in the spermatoduct spawning takes place. Note: when a testes is in the spent stage residual spermatozoa can be present in the spermatoduct. This will however be low numbers and the only other stage present will be spermatogonia.

Females

For females, maturity was determined in two ways (van Damme, 2022):

- By determining the proportion of oocytes and other structures at each development stage (Table 2.4) in the examined frames. This proportion was determined in two ways:
    - the surface proportion in the frames,
    - the proportional number of oocytes and structures in the frames.
- By determining the diameter of the largest oocytes and eggs at each development stage in the examined frames. (When the core is visible the oocyte diameter can be reliably measured, in some stages (e.g. migratory nucleus, hydrated) the core is not always visible, measuring the largest oocytes in these stages will provide the most reliable estimate.)

Table 2.4. Oocyte development stage and other structures descriptions (female fish)

Oocyte development stage	Explanation
PreVit	Previtellogenic oocytes, not developing
YV	Early developing oocytes, yolk vesicles present
YV-YG	Developing oocytes, yolk vesicles and yolk granules present
YG	Developing oocytes, yolk granules present
MIG	Late developing oocytes, migratory nucleus
HYD	Late developing oocytes, hydration stage, just before spawning
Eggs	Egg, spawned eggs, outer chorion is broken or has disappeared
POF	Post ovulatory follicle, the outer chorion that remains after the egg has been spawned. Will be resorbed by the female
Atr YV	Early atretic oocyte in the yolk vesical stage. Atretic cells are being resorbed by the female and will not further develop
Atr YV-YG	Early atretic oocyte in the yolk vesical- yolk granule stage
Atr YG	Early atretic oocyte in the yolk granule stage
LAtr	Late atretic oocytes
Massive atresia	More than 95% of the vitellogenic oocytes are atretic

Let "Na X" be the number of oocytes or structures per square centimetre at development stadium "X". And let "Vi X" be the surface proportion of the oocytes or structures at development stadium "X"[1]. The proportion of oocytes at each development stadium in the examined frames can thus be expressed in terms of Vi or Na at a development stadium. The Na and Vi variables use slightly adjusted definitions of the egg cell stadia (Table 2.5).

The presence of oocytes and structures is presented in these two ways because the size of the oocytes in the subsequent development stages is markedly different, previtellogenic oocytes are smaller than 185µm, while hydrated oocytes and eggs are around 1000µm.

Table 2.5. Explanation of the oocyte development stages as used for the proportion variables (female fish).

Term	Explanation
Na Vitellogenic	Total number of vitellogenic oocytes (YV, YV-YG, YG, MIG and HYD stages) per cm^2
Na Eggs	Total number of eggs per cm^2
Na POFs	Total number of POFs per cm^2
Na Alpha Atretic	Total number of early alpha atretic cells per cm^2
Na Latr	Total number of late atretic cells per cm^2
Vi PreVit	Proportion of previtellogenic oocytes by area
Vi Vitellogenic	Proportion of vitellogenic oocytes by area
Vi Eggs	Proportion of eggs by area
Vi POFs	Proportion of POFs by area
Vi Alpha atretic	Proportion of early alpha atretic cells by area
Vi Latr	Proportion of late atretic cells by area
Vi Wall	Proportion of ovary wall by area

[1] The proportions of Vi do not add up to 1, because negative grid (area outside the ovary) and space (due to the fixation, empty areas appear in the ovary) has not been taken into account

2.2 Data

The histological analysis resulted in three datasets, all used for the statistical analysis.

- Screening data: containing the presence/absence of the oocytes, structures and sperm cells at the different stadia.
- Weibel data: containing the "Na" and "Vi" variables of the oocytes and structures at the different stadia (see Table 2.5).
- Diameter data: containing the measurements of the diameters of the oocytes and eggs by development stage.

Each of these datasets also contains all the basic information regarding the fish:

- catch location; either in the northern or southern North Sea
- species (*Solea solea* or *Pleuronectes platessa*)
- fish length (cm), measured 'to the mm below'
- fish weight: total weight (g) of the fish, including all the organs and gonads, in grams
- gonad weight: weight (g) of only the gonads of the fish
- GSI: the gonadosomatic index, defined as gonad weight / (fish weight - gonad weight)
- K: Fulton's K, defined as 100 * fish weight / length(cm)^3

2.3 Statistical

The goal of the statistical analyses is to model the maturity over the months, correcting for some other covariates (like GSI), using oocyte and egg proportion and diameter as the responses.

2.3.1 Software

R version 3.6.3 (R Core Team, 2020) and R studio version 1.3.959 (RStudio Team, 2020) were used as the main software for the statistical analyses.

The `twosamples` (Dowd, 2020) R-package was used to perform the Bootstrapped two-sample Anderson-Darling and Kolmogorov-Smirnov tests.

2.3.2 Test for spatial differences

For both plaice and sole it is assumed that homing spawning behavior takes place during their respective spawning season, meaning that fish return to the spawning ground they origin from, in line with other species (Van Damme *et al*., 2009). The fish were sampled from both the northern and the southern part of the North Sea. For plaice the border was set at 53°N and for sole at 52°N to separate the spawning components. It should be noted that outside the spawning season the northern and southern fish can aggregate together, hence there is no means to be able to separate these spawning components outside the spawning season.

Since the number of sampled fish is relatively low, combining all fish would increase the sample size of the statistical analyses and thus the statistical power. But if the fish from the two areas are significantly different in biological parameters (length, weight, GSI and Fulton's K) from each other, they cannot responsibly be analysed together. Therefore, before performing the main analyses, it had to be determined whether the fish from the northern and the southern North Sea were similar enough to be analysed together, or too different to perform a robust pooled analysis.

"Different" here is not restricted to merely a difference in the mean value; any notable difference in the distribution (difference in mean, variance, quantiles, or skewness) was considered to be a relevant difference. So to test for a difference in this sense, a two-sample Bootstrapped Anderson-Darling Test

(Anderson & Darling, 1952, 1954; Stephens, 1974, 2017), a Bootstrapped two-sample Kolmogorov-Smirnov Test, and the Mann-Whitney U Test were performed. These tests were performed for every combination of species and sex, for a difference in GSI, Fulton's K, length, and weight (so each 16 sets of each test, 48 tests in total). Moreover, boxplots and density plots were prepared and compared between the fish of the North and of the South.

2.3.3 Models

Overview

No statistical models were run on the data of male fish, as only the screening for presence or absence of sperm cell development stages was carried out. Thus the below models were only run on the female data. The basic idea of all the models in this study was as follows: Take a measurement (diameter or proportion) of oocytes and eggs at a certain stage as the response variable, and model it against the months and GSI.

Based on the quality from a statistical perspective, it was decided to use a quasi-binomial GLM (logit link function) with the Na of a certain cell type as the response, and the sum of Na of all cell type as the binomial totals.

For the diameter models, gamma GLMs with log-link function was used, with the diameter of a certain cell type as the response. These models were performed only for females, separately by cell type, area and species.

Selected subsets

For most oocytes and egg stadia, no models could be run due to lacking sample size, and/or instances of perfect separation (the latter is only the relevant for the proportional count models). Therefore, for the proportional count models, only the "Previt" and "Vitellogenic" stadia were used. And for the diameter models, only the "PreVit", "YV", "YV-YG", and "YG" stadia were used.

Covariates

The months were entered as a B-spline, and GSI as a regular linear covariate into all models. For ease of interpretation, the models were run without an intercept term (recall that the B-spline for the months already included its own intercept).

As the number of individuals was not constant throughout the year, no time variable (month) was introduced as a categorical covariate (which would a-priori be the most obvious choice), but month was instead entered into the model as a B-spline (details in Annex 1).

GSI was chosen to be included in the models, as GSI is most directly related to maturity. The goal is to model the change of the diameter or proportion over the months, while correcting for length, fish weight, Fulton's K, and/or GSI. Unfortunately, certain combinations length, fish weight, Fulton's K, and GSI are often highly correlated (which combinations of variables are most correlated depends on the catch locations and species of the flatfish). Therefore, only one of these variables can be added to the model. The covariate GSI was entered a linear covariate in all models. This also keeps all models comparable and consistent. The number of observations was often too low for the proportion models to responsibly enter GSI as some form of spline. In the diameter models, GSI was found to behave reasonably linear. Moreover, due to the rather skewed distribution of GSI, entering GSI as some form of spline or polynomial may lead to over-fitting and/or deceptive extrapolation.

Diagnostics

The model fit to the data was checked in 2 ways:

- A scatterplot was produced with the observed diameter or proportion on the y-axis, and the predicted diameter or proportion on the x-axis. A straight line was fitted through these points, and the slope of this line (the "fit slope") was determined. The closer the fit slope is to 1, the better.
- The mean absolute deviation ("MAD") was calculated between the observed diameter or proportion and the predicted proportion. The closer the MAD is to zero, the better.

Residual diagnostic plots were produced to check for violations of the model assumptions. For the proportion models (quasi-binomial GLMs) deviance residuals were used, and for the diameter models (Gamma GLMs), Dunn-Smyth residuals (Dunn & Smyth, 1996) were used.

Variance structure

Due to the observed presence of heteroskedasticity in the diagnostic plots of some models, and because of the (theoretical) possibility of heteroskedasticity (like due to clustering), heteroskedasticity consistent standard errors (MacKinnon & White, 1985) were applied, using the sandwich (Zeileis, 2006; Zeileis, Köll, & Graham, 2020) and lmtest (Zeileis & Hothorn, 2002) R-packages. For consistency, these were used in all models. For the proportion models, HC3-type standard errors were used, and for the diameter models, HC1-type standard errors were used.

3 Results

3.1 Test for spatial difference

In order to test if there was a need to process the histological data by region (North/South), three tests were performed, for the male and females fish separately. If one of the tests shows a significant difference for one of the parameters analysed, it may be assumed that there is a spatial difference in some biological characteristics for that species. As a consequence, the histological data then have to be analysed separately for the fish from the northern and from the southern North Sea. Only the tabular outcomes are presented in the report. The boxplots and density plots visualizing these comparisons, can be provided by the authors upon request.

3.1.1 Males

For male sole, statistically significant differences occur between the northern and southern North Sea for fish length, fish weight, and GSI (Table 3.1, Annex 2) in the bootstrapped Anderson-Darling tests. For plaice, no significant spatial differences for males could be detected. In figures the north and south split has been made, even for male plaice, for consistency reasons. The results of the bootstrapped two-sample Kolmogorov-Smirnov test and the Mann-Whitney U test do not show any significant differences for other values (Annex 2; Tables A2.1, A2.2).

Table 3.1. Results for the males*: the Bootstrapped Anderson-Darling Tests comparing the distributions of GSI, Fulton's K, length, and weight between the North and South. SMD is the scaled mean difference, defined as (E(North)-E(South))/sd([North, South]). For the boxplots and density plots visualizing these comparisons, the reader is referred to the Supplementary Materials.*

variable	species	statistic	p-value	SMD	median(N) - median(S)	sd(N) / sd(S)
fish length	*Pleuronectes platessa*	0.04038	0.7769	0.07916	0.500000	1.3470
fish length	*Solea solea*	0.28810	0.0777 •	0.95760	3.500000	0.9533
fish weight	*Pleuronectes platessa*	0.05777	0.8730	-0.04863	-4.500000	1.2230
fish weight	*Solea solea*	0.46470	0.0791 •	0.66500	63.000000	0.8765
GSI x100	*Pleuronectes platessa*	0.24380	0.1879	-0.22650	0.109600	0.5452
GSI x100	*Solea solea*	0.75960	0.0123 *	-0.89740	-0.247600	0.3590
K	*Pleuronectes platessa*	0.17340	0.3418	-0.34310	-0.050480	1.5250
K	*Solea solea*	0.16590	0.5238	-0.56530	-0.007518	0.4481
significance code: 0 '***' 0.001 '**' 0.01 '*' 0.05 '•' 0.1 ' ' 1						

3.1.2 Females

For female sole, statistically significant differences occur between the northern and southern North Sea for fish length, fish weight, and GSI (Table 3.2). For female plaice, significant spatial differences for K occur (Table 3.2). As a consequence, for both species the histological data will be analysed separately for samples from the northern and the southern North Sea respectively. The results of the bootstrapped two-sample Kolmogorov-Smirnov test and the Mann-Whitney U test do not show any significant differences for other values (Annex 2; Tables A2.3, A2.4).

***Table 3.2. Results for the females**: the Bootstrapped Anderson-Darling Tests comparing the distributions of GSI, Fulton's K, length, and weight between the North and South. SMD is the scaled mean difference, defined as (E(North)-E(South))/sd([North, South]).*

variable	species	statistic	p-value	SMD	median(N) - median(S)	sd(N) / sd(S)
fish length	*Pleuronectes platessa*	0.04623	0.4880	0.1224	0.00000	0.7800
fish length	*Solea solea*	0.10360	0.0720 •	0.5436	3.50000	1.1800
fish weight	*Pleuronectes platessa*	0.11710	0.1735	0.1803	205.00000	0.8775
fish weight	*Solea solea*	0.19730	0.0691 •	0.5865	177.00000	1.2870
GSI x100	*Pleuronectes platessa*	0.04582	0.6859	0.2146	-0.08231	2.2910
GSI x100	*Solea solea*	0.19510	0.0744 •	0.4863	7.13100	1.2120
K	*Pleuronectes platessa*	0.20180	0.0457 *	0.5220	0.04367	1.0050
K	*Solea solea*	0.07155	0.4895	0.1742	0.03428	0.8472
significance code: 0 '***' 0.001 '**' 0.01 '*' 0.05 '•' 0.1 ' ' 1						

3.2 Histological information

3.2.1 Males

The proportion presence of the different development stages of sperm cells by species and geographic area (north/south) (Figure 3.1) does not show a completely consistent pattern for plaice (left panels in the figure) in relation to the main spawning period of the species (December-February). Spermatozoa in the spermatoduct are expected during spawning, and appear in July, November, March (south) and September (north). The appearance of spermatozoa in the spermatoduct is however supported by field observations during the beam trawl survey in 2021 (September), where three male plaice were encountered with sperm running from the milt.

For sole (Figure 3.1, right panels), the occurrence of well-developed sperm cells can only reasonably be evaluated for the southern North Sea, and seems to be in line with the commonly accepted spawning period for the species (March-June).

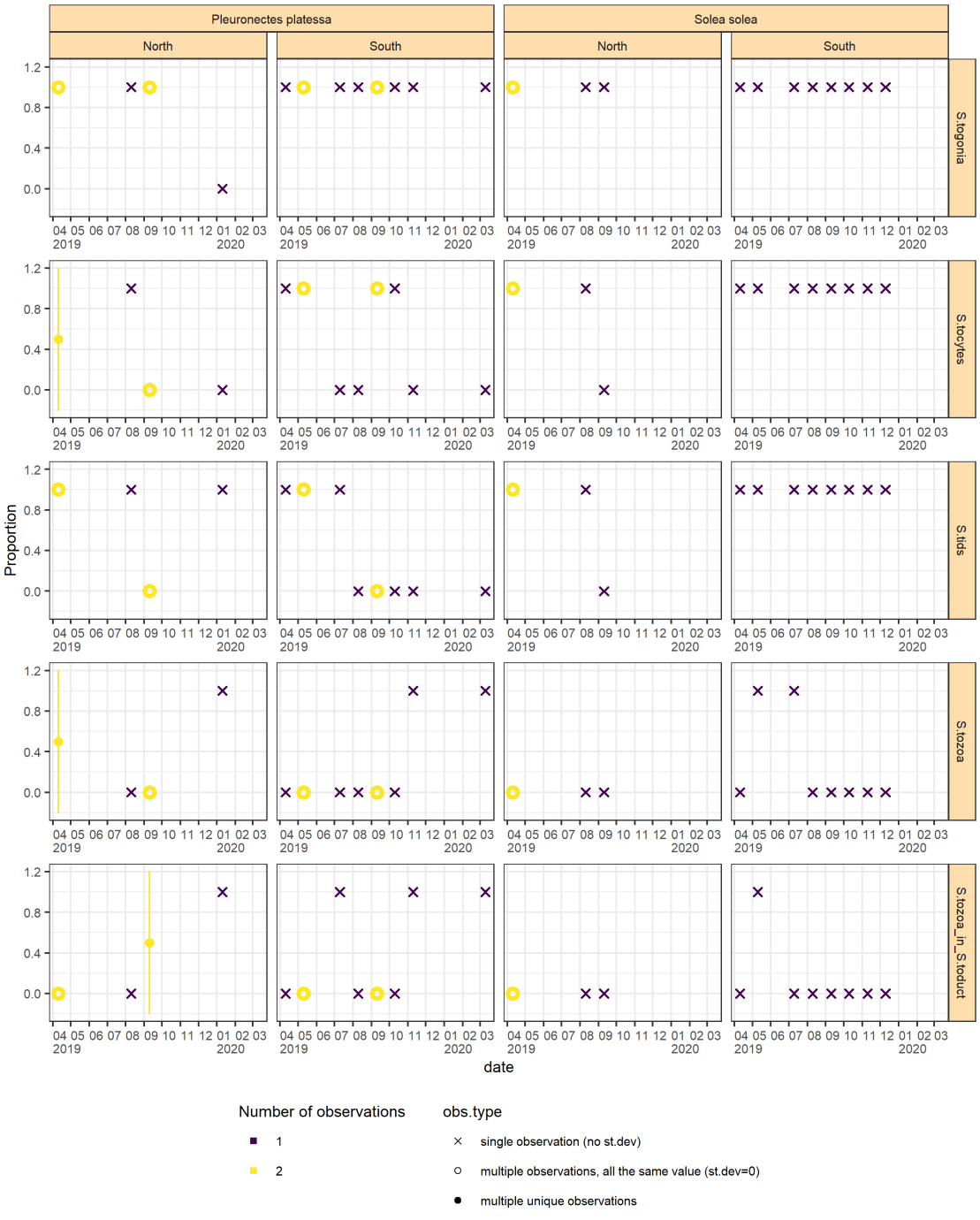


Figure 3.1. *Proportion presence of sperm cells by species, and geographic area (north/south) and development stage. Points indicate means, lines indicate standard deviations (st.dev). If there is only one observation for a group, no standard deviation can be computed (indicated by the "X")*

3.2.2 Females

The development of the female histological maturity can easiest be evaluated by the development of the relative number oocyte types per cm^2 (Table 3.3, Figure 3.2), and the oocyte diameter of the various development stages (Table 3.4, Figure 3.3).

For the predicted estimates (Figures 3.2 and 3.3), GSI was set to the mean value (indicated inside the plot), and predictions were made as the months vary. The points indicate the means, the shaded ribbon and vertical lines indicate the 95% confidence interval. When for the predictions the 95% confidence interval of month A does not reach the mean value of month B, and vice-versa, the months can be said to be significantly different. The plots of the actual data are presented in Annex 3.

During the histological analyses it was observed that for plaice the migratory nucleus stage only appeared in the middle to the end of the spawning season (in January), and hydrated eggs were only encountered in January/February, towards the end of the spawning season. Only in sole in the southern North Sea atresia was observed. No massive atresia was observed.

Proportional counts

The model predictions for the proportional count of the different oocyte types were only good for sole vitellogenic oocytes in the southern North Sea. It means that it is very difficult to predict the proportional count of an oocyte type in the next month for sole in the southern area and for plaice. This is partly due to the low number of observations (gonads) in this study.

Data for plaice in the northern North Sea (Figure 3.2 left hand figures) are too limited to provide insight in the development of the oocyte types over time. A relatively high proportion of vitellogenic oocytes occurs for plaice in the southern North Sea (Figure 3.2 second column of figures) from June till August, followed by a decline of vitellogenic oocytes in the months after and an increase towards the known spawning season. This may indicate that there is food limitation and fish is resorbing vitellogenic oocytes. This is however not supported by atresia recordings (Annex 3, figure A3.1, 2nd column of figures, lower two rows). On the other hand, spawning activity may have taken place in the southern North Sea in that period. The presence of post ovulatory follicles (POFs) in the samples from September to November 2019 (Annex 3, figure A3.1, 2nd column of figures, 4th row) supports that assumption.

For sole in the northern North Sea (Figure 3.2 third column of figures) data for some months are missing, but the data from the other months follow a similar pattern as for sole in the southern North Sea (Figure 3.3. right hand figures). There, oocyte development takes place according to expectation: an increase of the proportion of vitellogenic oocytes towards the spawning season, and an increase of the previtellogenic oocytes after the spawning season.

Table 3.3. *Table reporting for each proportional count model the number of observations it was based on, the dispersion, Mean Absolute Deviation (MAD, closer to zero is better), fit slope (closer to 1 is better)*

response	species	area	n	family	dispersion	MAD	fitslope
na_previt	*Pleuronectes platessa*	North	14	quasibinomial	63.94149	0.14522302	0.5828847
na_previt	*Pleuronectes pletessa*	South	18	quasibinomial	55.77330	0.15988918	0.8006696
na_previt	*Solea solea*	North	10	quasibinomial	190.30551	0.09314022	0.8501802
na_previt	*Solea solea*	South	22	quasibinomial	96.57893	0.11686634	1.2643800
na_vitellogenic	*Pleuronectes platessa*	North	14	quasibinomial	35.25125	0.09336274	0.6548260
na_vitellogenic	*Pleuronectes pletessa*	South	18	quasibinomial	51.09165	0.16444806	0.3164744
na_vitellogenic	*Solea solea*	North	10	quasibinomial	186.18517	0.10233343	0.7969137
na_vitellogenic	*Solea solea*	South	22	quasibinomial	91.08617	0.09614576	1.0589834

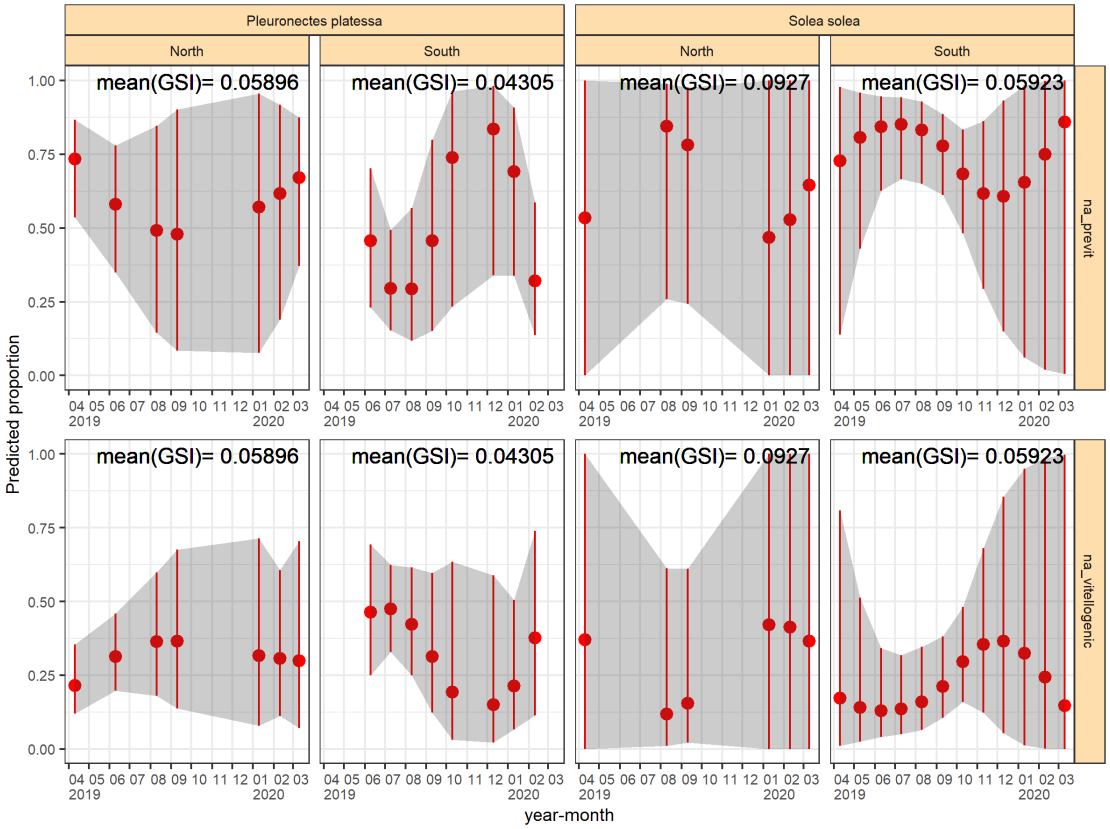


Figure 3.2. *Proportional counts: Predictor Effect plots of the months' effect in the quasi-Binomial GLMs. Each plot gives this effect for a specific combination of location (North/South), species, and oocyte type. The points indicate the means, the shaded ribbon and vertical lines indicate the 95% confidence interval.*

<u>Oocyte diameter</u>

The model predictions for the diameters are quite good for both species for all development stages, in the northern as well as the southern North Sea (Table 3.4, Figure 3.3). The number of observations is quite high as all cells are measured individually.

For plaice in the northern as well in the southern North Sea (Figure 3.3 first and second column of figures) the diameters of the egg cells develop according to the expectation that the oocytes increase in size throughout the year and towards the spawning season, including the shift from the early development stage to later stages.

For sole in the northern North Sea (Figure 3.3 third column of figures) the increase of previtellogenic oocyte diameter in August/September, and the relatively small diameter of vitellogenic oocytes is expected to be caused by oocytes just on the edge of turning from previtellogenic into vitellogenic oocytes. The diameter size patterns of the other oocyte stages are in line with the expectations based on the timing of the spawning season. For sole in the southern North Sea (Figure 3.3. right hand figures), diameter size development follows the expected curve: increasing diameters towards the spawning season.

Table 3.4. *Table reporting for each diameter model the number of observations it was based on, the dispersion, Mean Absolute Deviation (MAD, closer to zero is better), fit slope (closer to 1 is better)*

response	species	area	n	Family	dispersion	MAD	fitslope
YV	*Pleuronectes platessa*	North	71	Gamma	0.02634348	0.02664751	0.9809158
YV	*Pleuronectes pletessa*	South	113	Gamma	0.03667791	0.03214024	0.9982883
YV	*Solea solea*	North	84	Gamma	0.06824853	0.04192031	0.9820191
YV	*Solea solea*	South	172	Gamma	0.03703105	0.03081160	0.9865182
PreVit	*Pleuronectes platessa*	North	110	Gamma	0.02518468	0.01594731	1.0010361
PreVit	*Pleuronectes pletessa*	South	153	Gamma	0.07131326	0.02795948	1.0221025
PreVit	*Solea solea*	North	109	Gamma	0.04698610	0.01696313	0.9922627
PreVit	*Solea solea*	South	211	Gamma	0.03765182	0.01660407	1.0324957
YV-YG	*Pleuronectes platessa*	North	65	Gamma	0.01698099	0.03033180	0.9641410
YV-YG	*Pleuronectes pletessa*	South	81	Gamma	0.02804380	0.04291485	0.8804795
YV-YG	*Solea solea*	North	88	Gamma	0.03418615	0.05557687	0.9857342
YV-YG	*Solea solea*	South	136	Gamma	0.07007754	0.09374258	0.9767179
YG	*Pleuronectes platessa*	North	41	Gamma	0.01685435	0.05483841	1.0010385
YG	*Pleuronectes pletessa*	South	62	Gamma	0.02015117	0.06207884	0.9990977
YG	*Solea solea*	North	70	Gamma	0.01365582	0.05953415	1.0000017
YG	*Solea solea*	South	51	Gamma	0.01473934	0.06647974	1.0347049

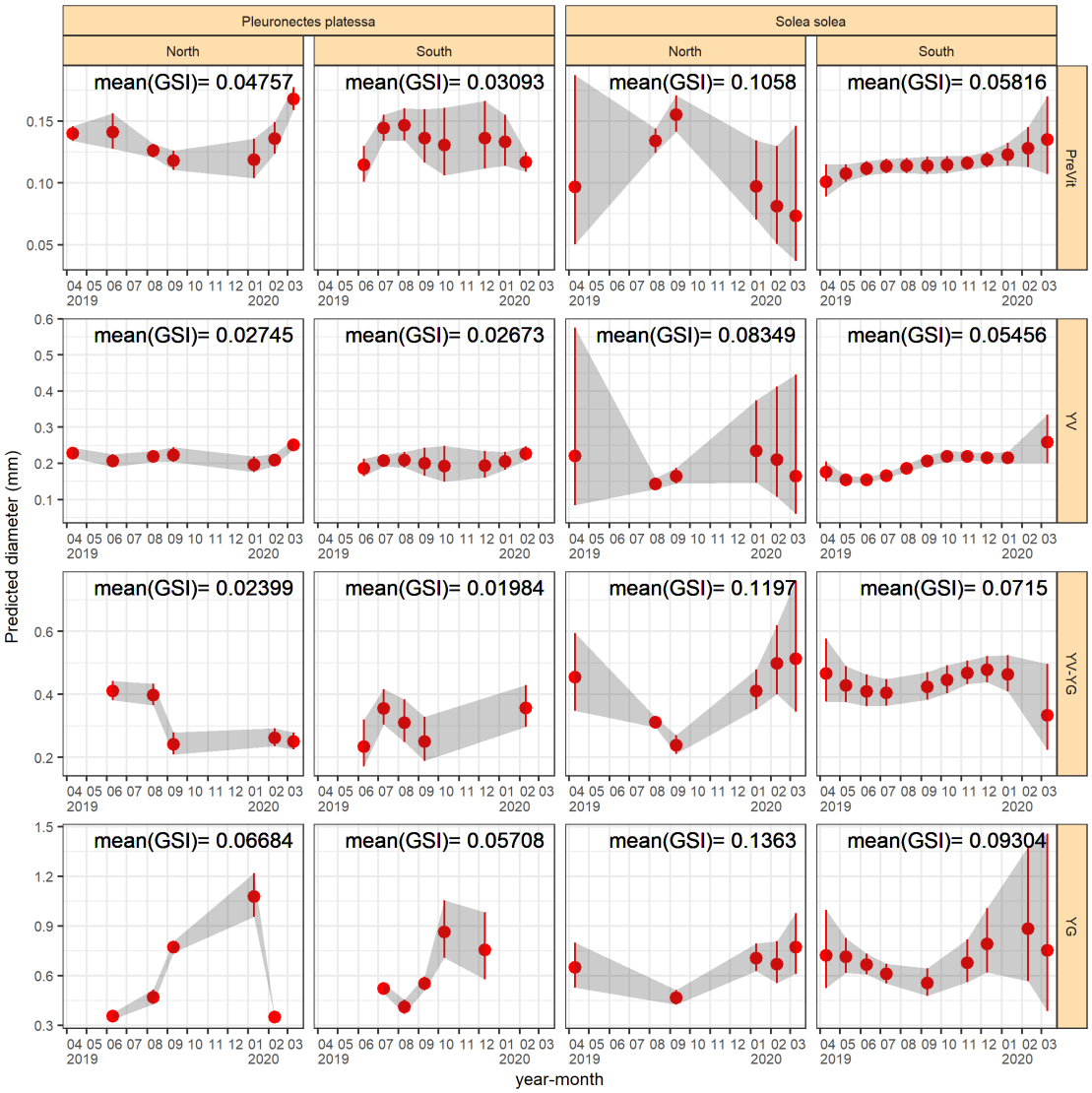


Figure 3.3. *Oocyte diameter: Predictor Effect plots of the months' effect in the gamma GLMs. Each plot gives this effect for a specific combination of location (North/South), species, and oocyte type. The points indicate the means, the shaded ribbon and vertical lines indicate the 95% confidence interval.*

4 Conclusions and discussion

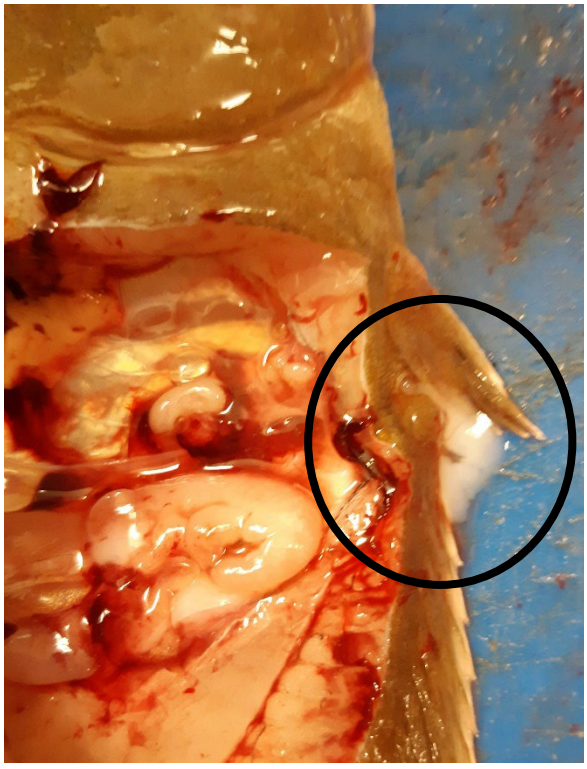
4.1 Maturity development throughout the year

It is hard to statistically underpin the maturation over time. This is largely due to the low number of data, making it difficult to model the development of cell types, especially the previtellogenic stage towards the vitellogenic stages. The low number of data is not caused by the lack of samples collected, but due to the fact that conducting histological analyses is labour intensive. Partly this is because the gonads have to be transformed into physical slides, and pictures have to be taken from those slides. The analysis of the slides, i.e. identifying development stages, counting oocytes, measuring oocyte diameter, requires however the largest effort in the process and can be considered as the main factor determining the time needed for the work. Image analysis and development of artificial intelligence (AI) algorithms to automatically identify the different oocyte development stages may be a way to increase the amount of samples that can be analysed in a certain timeframe, and lead to increased statistical underpinning of results. Currently automatization of histological samples is being developed, in cooperation with colleagues at Ifremer (France).

The oocyte diameter increases during the maturation and following spawning cycle, from a small diameter after the previous spawning season, towards larger diameters as the nest spawning season comes closer. The diameters can reliably be modelled over time, for both plaice as well as sole.

4.2 Observed versus expected development

The expected development for both species is that the relative number of vitellogenic oocytes and their diameters increase towards the spawning season, and decline soon afterwards. As a consequence, the relative number of previtellogenic oocytes is expected to increase soon after the end of the spawning season, when the maturation cycle starts again.



4.2.1 Plaice

The plaice development seems to be aberrant from the expectation, especially in the southern North Sea. The relatively high proportion of vitellogenic oocytes from June till August, followed by the decline and an increase towards December is not in line with the expectation. Plaice, as a capital winter spawner, is to be expected to build up the number of oocytes and let them evolve gradually towards the spawning season. The decline of the relative number of vitellogenic oocytes is most likely due to spawning activity, as post ovulatory follicles (POFs) were encountered in the samples from September to November 2019. Incidental spawning activity in summer for plaice in the southern North Sea is in line with the signals from the North Sea BTS in August 2018 (female with well-developed gonad), and September 2021 (males with running milt, see circle in picture).

A study of plaice gonad development in 2003 and 2004 showed that plaice was able to up-regulate fecundity in summer (van Damme, 2013). New oocytes were being recruited from the previtellogenic

stock during the summer period. However, no evidence of the presence of hydrated oocytes or spawned eggs were found in 2003-2004. It does however show that plaice is able to invest high amounts of surplus energy in reproduction in the summer period when there is plenty of food available. The flexibility in oocyte development was already present in 2003 and 2004 and the step to utilise this surplus energy to actually speed up oocyte development and spawning in summer time might not be huge.
The maturation of sole -most explicit in the southern North Sea- seems to be in line with the expectations that previtellogenic oocytes are replaced by vitellogenic cells that develop into eggs during the spawning season.

4.2.2 Sole

For sole no changes in the maturity pattern were found in this study, despite reporting from market sampling that sole gonads looked like developing from the expected pattern. The number of fish analysed in this study are low, due to the time-intensive labour of the histological analyses. Possibly the low number of samples missed any aberrant development in sole. However, analyses of GSI and Fulton's K development over the year in the time-series from 1996 to 2019 did not show any changes over time (Chen *et al*. 2019, Wilkes 2020). This supports the results of the current study that no changes in gonad maturation are found.

4.3 Effect on population level

4.3.1 Plaice

It is currently unclear what the effect of this on the plaice population is. The fact that both males and females seem ready to spawn in the summer, indicates that spawned summer eggs are probably fertilized. However, it is unknow what the quality of these eggs is, if larvae hatch from them, and if those larvae survive the winter period. Furthermore, these summer-spawning plaice were not caught on the traditional spawning grounds and therefore it might be that the larvae from these eggs do not (all) reach the traditional nursery areas. On the other hand high numbers of extremely small young-of-the-year plaice were found in autumn in the Dutch Wadden Sea (Beier *et al*., 2022) in 2021. If these originated from the winter 2020-2021 spawning event growth was very limited for these specimens. It could be possible that these originate from the summer spawning event.

Previous results of analyses of biological parameters of the historic market sampling for plaice showed that from 1996 to 2018 no changes in GSI or Fulton's K development over the year were found (Chen *et al*. 2019, Wilkes 2020). But 2019 showed a significant difference in GSI and Fulton's K development over the year compared to the earlier data (Wilkes 2020). It is therefore likely that before 2018 summer spawning did not take place, whereas from 2019 onwards both this study and results from the BTS surveys indicate summer spawning. Further investigation is needed if this summer spawning is increasing and if these summer larvae will recruit to the adult stock.

4.3.2 Sole

As no changes in the maturation pattern of sole gonads were found in this study and analyses of GSI and Fulton's K development over the year in the time-series did also not show changes, no effect on the sole population is expected.

5 Quality assurance

5.1 Histological analysis

A ring test was carried out between the experts analysing the histological samples. The results were discussed between the experts.

For staging of histological slides from male fish, a manual was developed throughout the project (Blom, 2022).

5.2 ISO 9001:2015

CVO is certified to ISO 9001:2015 (certificate number: 268632-2018-AQ-NLD-RvA). This certificate is valid until December 15th, 2024. The certification was issued by DNV Business Assurance B.V**.**

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Justification

CVO Report: 22.001
Project number: 4311300073

The quality of this report has been peer reviewed by a colleague scientist and the head of CVO.

Approved by: Dr. P.J.A. de Bruijn
onderzoeker

Signature: 

Date: March 23, 2022

Approved by: Ing. S.W. Verver
Head Centre for Fisheries Research

Signature: 

Date: March 23, 2022

Annex 1 Details on B-spline

Details on the B-spline used for the month effect in the models

Let yearmonth_num be a variable defined as follows: When the year is 2019, yearmonth_num is the month number; when the year is 2020, yearmonth_num is the month number **+ 12** (i.e. 4="April 2019", 5="May 2019", 13="January 2020", etc.). Then, yearmonth_num was entered in all the models as a B-spline (including its intercept) with boundary knots at 0 and 15, and one internal knot at 9.5. The degree of this B-spline was second degree in the proportional models, and third degree in the diameter models (a lower degree in the proportion models as it has too few observations to handle a third degree spline).

Additional software extensions used

The Bookdown (Xie, 2016) extension of R-Markdown (Allaire *et al*., 2019) was used for the statistical reporting.

The R-packages ggplot2 (Wickham, 2016), ggh4x (van den Brand, 2021), viridis (Garnier, 2018) and flextable (Gohel, 2021) were used for the visualizations.

Annex 2 Additional tests for spatial difference

Table A2.1. *Results of the Bootstrapped Kolmogorov-Smirnov Tests comparing the distributions of GSI, Fulton's K, length, and weight between the North and South for the males of both species. SMD is the scaled mean difference, defined as (E(North)-E(South))/sd([North, South]).*

variable	species	statistic	p-value	SMD	median(N) - median(S)	sd(N) / sd(S)
fish length	*Pleuronectes platessa*	0.1667	0.9378	0.07916	0.500000	1.3470
fish length	*Solea solea*	0.5000	0.2902	0.95760	3.500000	0.9533
fish weight	*Pleuronectes platessa*	0.1667	0.9717	-0.04863	-4.500000	1.2230
fish weight	*Solea solea*	0.6250	0.1647	0.66500	63.000000	0.8765
GSI x100	*Pleuronectes platessa*	0.4000	0.3570	-0.22650	0.109600	0.5452
GSI x100	*Solea solea*	0.7500	0.0588 •	-0.89740	-0.247600	0.3590
K	*Pleuronectes platessa*	0.3667	0.4902	-0.34310	-0.050480	1.5250
K	*Solea solea*	0.3750	0.7006	-0.56530	-0.007518	0.4481
significance code: 0 '***' 0.001 '**' 0.01 '*' 0.05 '•' 0.1 ' ' 1						

Table A2.2. *Results of the Mann-Whitney U Tests comparing the distributions of GSI, Fulton's K, length, and weight between the North and South for the males of both species. SMD is the scaled mean difference, defined as (E(North)-E(South))/sd([North, South]).*

variable	species	statistic	p-value	SMD	median(N)-median(S)	sd(N) / sd(S)
fish length	*Pleuronectes platessa*	30	1.00000	0.07916	0.500000	1.3470
fish length	*Solea solea*	26	0.09926 •	0.95760	3.500000	0.9533
fish weight	*Pleuronectes platessa*	29	0.95669	-0.04863	-4.500000	1.2230
fish weight	*Solea solea*	24	0.20194	0.66500	63.000000	0.8765
GSI x100	*Pleuronectes platessa*	32	0.87488	-0.22650	0.109600	0.5452
GSI x100	*Solea solea*	5	0.07402 •	-0.89740	-0.247600	0.3590
K	*Pleuronectes platessa*	25	0.63536	-0.34310	-0.050480	1.5250
K	*Solea solea*	14	0.79856	-0.56530	-0.007518	0.4481
significance code: 0 '***' 0.001 '**' 0.01 '*' 0.05 '•' 0.1 ' ' 1						

Table A2.3. *Results of the Bootstrapped Kolmogorov-Smirnov Tests comparing the distributions of GSI, Fulton's K, length, and weight between the North and South for the females of both species. SMD is the scaled mean difference defined as (E(North)-E(South))/sd([North, South]).*

variable	species	statistic	p-value	SMD	median(N) - median(S)	sd(N) / sd(S)
fish length	*Pleuronectes platessa*	0.2460	0.4902	0.1224	0.00000	0.7800
fish length	*Solea solea*	0.4182	0.0782 •	0.5436	3.50000	1.1800
fish weight	*Pleuronectes platessa*	0.3413	0.1957	0.1803	205.00000	0.8775
fish weight	*Solea solea*	0.4182	0.1084	0.5865	177.00000	1.2870
GSI x100	*Pleuronectes platessa*	0.1746	0.8776	0.2146	-0.08231	2.2910
GSI x100	*Solea solea*	0.3273	0.3061	0.4863	7.13100	1.2120
K	*Pleuronectes platessa*	0.3968	0.0951 •	0.5220	0.04367	1.0050
K	*Solea solea*	0.2182	0.7339	0.1742	0.03428	0.8472
significance code: 0 '***' 0.001 '**' 0.01 '*' 0.05 '•' 0.1 ' ' 1						

Table A2.4. *Results of the Mann-Whitney U Tests comparing the distributions of GSI, Fulton's K, length, and weight between the North and South for the females of both species. SMD is the scaled mean difference, defined as (E(North)-E(South))/sd([North, South]).*

variable	species	statistic	p-value	SMD	median(N) - median(S)	sd(N) / sd(S)
fish length	*Pleuronectes platessa*	134.0	0.7753	0.1224	0.00000	0.7800
fish length	*Solea solea*	145.5	0.1527	0.5436	3.50000	1.1800
fish weight	*Pleuronectes platessa*	146.0	0.4588	0.1803	205.00000	0.8775
fish weight	*Solea solea*	143.0	0.1897	0.5865	177.00000	1.2870
GSI x100	*Pleuronectes platessa*	115.0	0.6941	0.2146	-0.08231	2.2910
GSI x100	*Solea solea*	128.0	0.4832	0.4863	7.13100	1.2120
K	*Pleuronectes platessa*	169.0	0.1071	0.5220	0.04367	1.0050
K	*Solea solea*	127.0	0.5087	0.1742	0.03428	0.8472

significance code: 0 '***' 0.001 '**' 0.01 '*' 0.05 '•' 0.1 ' ' 1

Annex 3 Figures from histological analyses

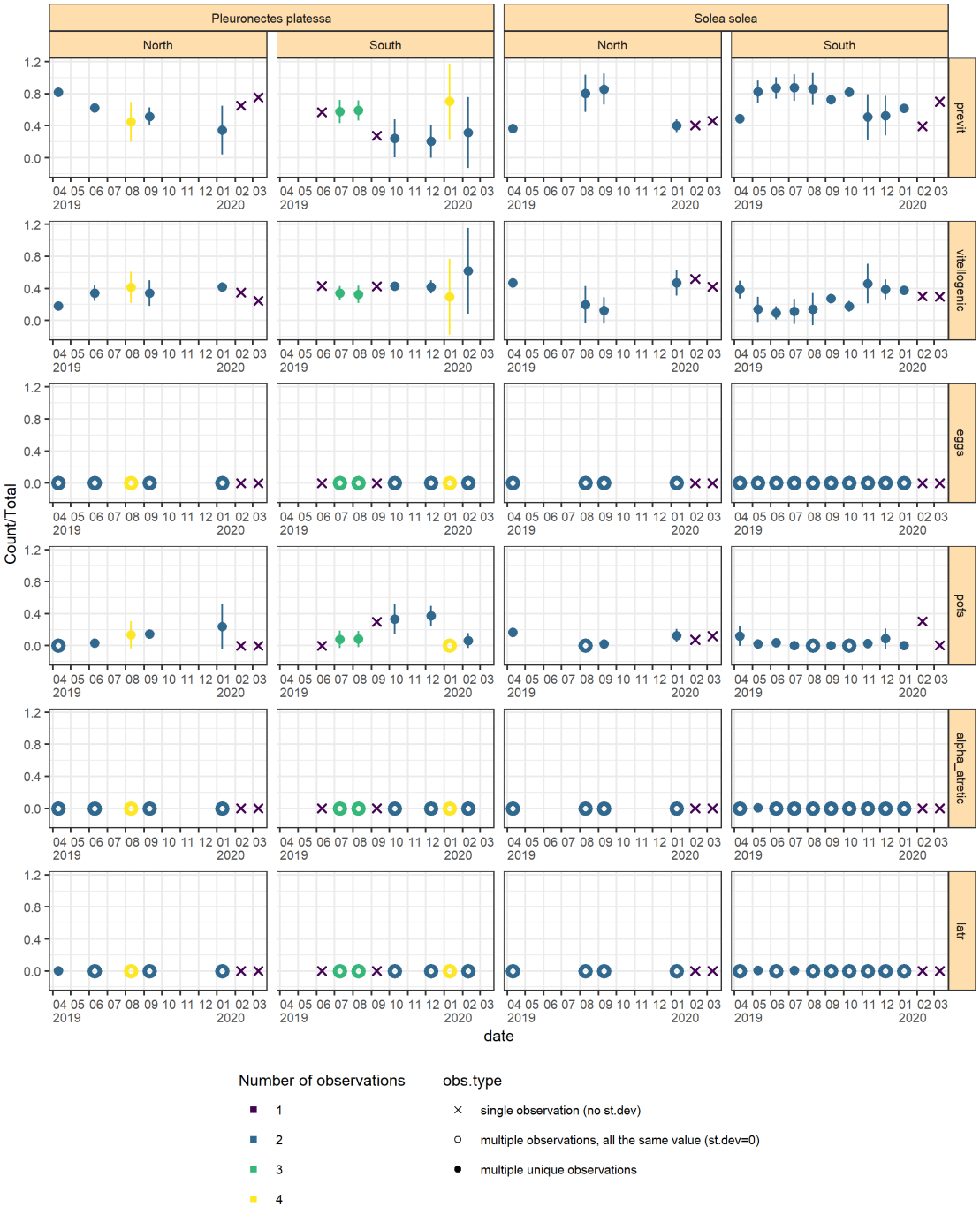

Figure A3.1. *Number of one cell type per cm² divided by the total number of cell types per cm². Points indicate means, lines indicate standard deviations (st.dev). If there is only one observation for a group, no standard deviation can be computed (indicated by the "X")*

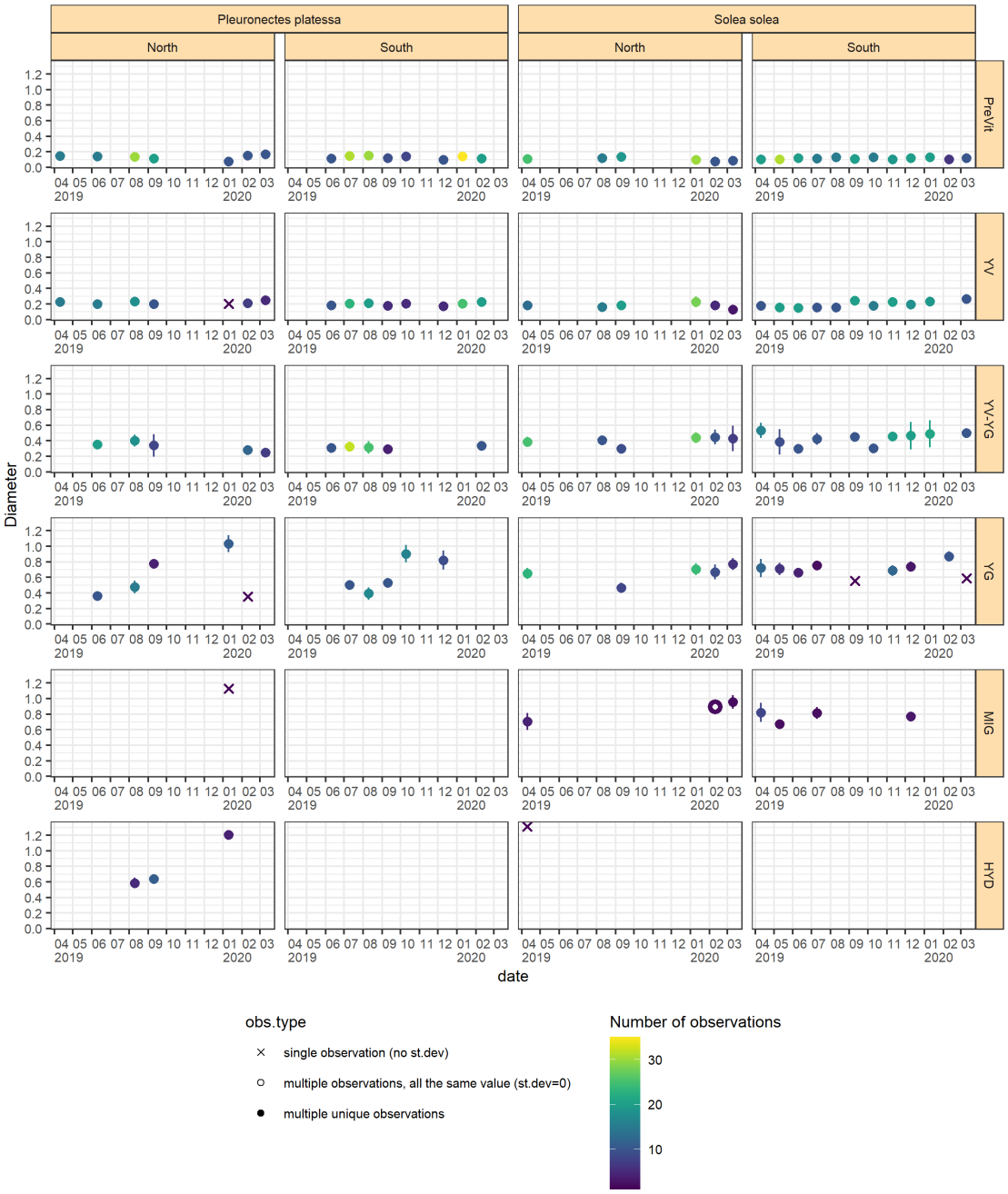

Figure A3.2. *Diameter of oocytes at various stages. Points indicate means, lines indicate standard deviations (st.dev). If there is only 1 observation for a group, no standard deviation can be computed (indicated by the "X")*