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SPECIAL SECTION: FISHERIES REPRODUCTIVE BIOLOGY

Quantification of Primary and Secondary Oocyte Production in Atlantic Cod by Simple Oocyte Packing Density Theory

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Abstract

As for other teleosts, the level of primary oocyte production ultimately determines the number of eggs shed by Atlantic cod *Gadus morhua*, but so far these minute cells have been little studied, probably due to methodological challenges. We established a quantitative "grid method" based on simple oocyte packing density (OPD) theory, accurate input data on ovary volume, oocyte-stage-specific ovarian volume fractions (from hits on grid-overlaid sections), and individual oocyte volumes (from diameter measurements of transections). The histological OPD results were successfully validated by automated measurements in whole mounts. The analyzed material originated from cultured Atlantic cod held in tanks for 19 months through the first maturity cycle and part of the second maturity cycle. Prior to sexual maturity, none of the fish showed the so-called circumnuclear ring (CNR; rich in RNA and organelles) in the cytoplasm of their primary oocytes, but this ring (phases 4a, 4b, and 4c) quickly appeared later on around the time of the autumnal equinox, followed by production of cortical alveolar oocytes (CAOs), early vitellogenic oocytes (EVOs), and late vitellogenic oocytes (LVOs). A very similar pattern was observed in the second maturity cycle. Thus, it is concluded that an autumnal night longer than 12 h generally triggers oocyte growth in Atlantic cod. A few immature individuals became arrested at the early CNR phase (phase 4a); hence, the use of CNR presence as a maturity marker should be treated with some caution. The maximum OPD was 250,000 oocytes/g of ovary for phase 4a; 100,000 oocytes/g for combined phases 4b and 4c; 100,000 oocytes/g for CAOs; 50,000 oocytes/g for EVOs; and 25,000 oocytes/g for LVOs. The relative somatic fecundity showed a dome-shaped curve with oocyte development (from CAO to LVO). Production of CAOs appeared at a fresh oocyte diameter of 180 μm, which is significantly below the commonly accepted threshold value of 250 μm for developing Atlantic cod oocytes.

Oogenesis in the Atlantic cod *Gadus morhua* has been addressed in many publications, but the main focus, at least within applied fisheries reproductive biology, has been on secondary growth (potential fecundity). Thus, few studies deal with primary growth, apparently because these very small cells are difficult to assess and are often considered to be present in superfluous numbers. Woodhead and Woodhead (1965) postulated that only those cells exhibiting the so-called circumnuclear ring (CNR; consisting mainly of organelles and RNA and assumed to be homologous with the Balbiani body; see Kjesbu and Kryvi 1989 and Zelazowska et al. 2007) in the cytoplasm by late autumn will complete oocyte maturation. This was further specified by Shirokova (1977) and Holdway and Beamish (1985) as applying to oocytes beyond the early CNR phase (the phases are described below). Tomkiewicz et al. (2003) questioned this view because CNR oocytes appeared throughout the year. The collective results of these studies suggest that there is still uncertainty about the size at which Atlantic cod oocytes should be considered as developing.

Over the last decade, significant progress has been made within applied fisheries reproductive biology in terms of oocyte characterization and quantification, as summarized by

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Witthames et al. (2009) and Kjesbu et al. (2010b). These advancements are partly the result of implementation of laboratory techniques already in place elsewhere and have been facilitated by the rapid development of digital image analysis. In particular, the adoption of the disector method (Sterio 1984) by marine laboratories (Andersen 2003; Kraus et al. 2008; Kjesbu et al. 2010a; M. Korta and H. Murua, AZTI Tecnalia, unpublished) has given access to unbiased numerical estimates from histological slides. Also important is the introduction of the autodiametric method (Thorsen and Kjesbu 2001; Klibansky and Juanes 2008; Alonso-Fernández et al. 2009), which allows developing oocytes to be quickly measured and counted. However, both the disector method and the autodiametric method have some intrinsic problems. The main argument against the disector method is the high labor cost involved, although various time-saving software programs do exist; for the autodiametric method, the main disadvantage is the insufficient ability to measure transparent oocytes (i.e., chromatin nucleolus and primary growth oocytes; Grier et al. 2009). Primary growth oocytes consist of previtellogenic oocytes (PVOs) and cortical alveolar oocytes (CAOs). In practice, the autodiametric method therefore works well for determinate spawners (with completed de novo oocyte recruitment) but less so for indeterminate spawners (with ongoing de novo oocyte recruitment; Witthames et al. 2009). The latter situation has led to the development of advanced oocyte packing density (OPD) theory, which combines information from both histology and image analysis (Kurita and Kjesbu 2009; Korta et al. 2010). Because in-depth algorithms are required when working with indeterminate spawners, such studies are rather sophisticated in nature. Thus, in this article we reduce the complexity of methods for estimating OPD in a determinate spawner, the Atlantic cod.

Ideally, to achieve a better understanding of the underlying history of primary oocytes, one should undertake unbiased calculations on a material with known history, such as samples obtained from aquaculture. Atlantic cod reared for mariculture (Rosenlund and Skretting 2006) are preferable because the de facto existence of spawning zones in otoliths in this species (Rollefsen 1934) has not yet been properly validated and because the use of postovulatory follicles (POFs) as a reliable long-term postspawning marker is relatively new (Saborido-Rey and Junquera 1998; Skjæraasen et al. 2009; Witthames et al. 2010). Therefore, the specific aims of the present article were to (1) conduct an experimental study of sufficient length to determine when the different oocyte stages recruit, (2) quantify primary and secondary oocyte production by using simple OPD theory, and (3) present an improved fecundity (F) regulation model.

METHODS

To the extent possible, Atlantic cod were maintained under natural conditions in terms of temperature, photoperiod, and food intake (detailed below). Because the fish originated from aquaculture, their previous history was well known. Also, as cultured Atlantic cod generally spawn for the first time at the age of 2 years (Karlsen et al. 1995), the experiment could be planned accordingly to cover the initiation of maturation (sexual maturity) from the immature phase through subsequent reproductive phases. We studied the complete first maturity cycle but ended the experiment just before the second spawning season. Thus, the body and ovary measurement program was undertaken on fish monitored over nearly two maturity cycles.

Background History of the Experimental Fish

All specimens were reared at the Institute of Marine Research field station Parisvatnet, a large marine pond system located west of Bergen, Norway (Blom et al. 1994; Otterå et al. 2006). These fish were the offspring of a local broodstock and therefore should be considered as Norwegian coastal Atlantic cod. Immediately after hatching in incubators during spring 2001, the larvae were introduced into the pond and were offered natural zooplankton. Juveniles and subsequent adolescent stages were fed various types of dry feed formulated for marine fish (Skretting, Stavanger, Norway). At the time of juvenile confinement in summer, all were dip-vaccinated against vibriosis prior to stocking into separate sea cages.

Main Experimental Set-up

Once the fish reached approximately 1 year of age and 400–500 g in body weight, a random subsample of fish was taken on 8 and 10 May 2002; these individuals were transported in oxygenated tanks to the main laboratory in Bergen. The fish were put into one of two neighboring, identical, 30-m^3 outdoor tanks (length = 6 m; width = 3 m; water depth = 1.65 m), which were labeled as tanks A and B (Table 1) and functioned as replicates. Seawater was pumped from 120-m depth in the fjord, was sand filtered and degassed, and was supplied to each tank at a rate of about 80 L/min. Each tank was covered by a net to moderate the light intensity by 70%. Feces and any waste feed on the tank bottom were removed by vacuum-cleaning once per week.

The experiment was run from 18 June 2002 to 8 January 2004 (569 d; Tables 1, 2). Initially, all fish were individually tagged with passive integrated transponder tags, weighed to determine whole-body weight (W_{body}; nearest 1 g), and measured for total length (L_{total}; nearest 0.5 cm). Thereafter, W_{body} and L_{total} were measured every 2–3 months until the end of the experiment (Table 2). During handling, all fish were anesthetized with benzocaine (60 mg/L) in oxygenated seawater (Kjesbu et al. 1991). A few fish did not recover from this anesthetic bath, died later, or were removed due to injuries. An additional number of individuals (tank A: $n = 11$; tank B: $n = 13$) that were fitted with data storage tags in January 2003 (Righton et al. 2006) were also excluded from the analyses as the effect on oocyte development rate was unknown.

The fish were hand-fed dry pellets (11–15 mm) of a special broodstock feed (DAN-EX 1758; Dana Feed [BioMar] A/S,

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TABLE 1. Feeding ration (FR; % dry feed · g body weight^{-1} · d^{-1}) and number (n) of Atlantic cod females and males in tanks A and B during the experimental study. The FR values for periods close to or during spawning are marked in bold. Mean FR and associated SD are given per tank. The fish were fed ad libitum until the end of October 2002; thereafter, they received a moderate ration.

	Tank A		Tank B	
Time period	n	FR	n	FR
May–Jun 2002[a]	202	Not available	222	Not available
Jun–Aug 2002	187	0.46	222	0.43
Aug–Oct 2002	178	0.36	214	0.31
Oct 2002–Jan 2003	154	0.25	191	0.23
Jan–Mar 2003	132	**0.10**	167	**0.12**
Mar–May 2003	108	**0.19**	146	**0.16**
May–Jul 2003	94	0.21	115	0.23
Jul–Sept 2003	60	0.25	89	0.27
Sep–Nov 2003	36	0.29	69	0.30
Nov 2003–Jan 2004	29	**0.17**	60	**0.13**
Mean (SD) across all time periods		0.25 (0.11)		0.24 (0.10)

[a]Acclimation period prior to start of experiment.

TABLE 2. Overview of the number of Atlantic cod females (n) that were sacrificed and studied by different types of laboratory methodology per experimental month (LC = Leading cohort). Data apply to both tanks. Hyphen reflects no data; parentheses indicate a missing sampling point. For each fish, two ovarian samples were obtained for histology: one was fixed in Bouin's fluid, and the other was fixed in formaldehyde. Sum is the total n sacrificed or analyzed.

			Oocyte characterization		Oocyte quantification	
Date	Experimental day	Sacrificed (n)	Fresh LC diameter (n)	Histology[b] (n)	Grid method (n)	Autodiametric method (n)
18 Jun 2002	0[a]	11	–	11	–	–
17 Jul 2002	29	10	–	9	3	–
28 Aug 2002	71[a]	10	–	10	5	–
26 Sep 2002	100	10	9	10	5	–
31 Oct 2002	135[a]	10	10	10	5	–
28 Nov 2002	163	10	10	10	6	–
(Dec 2002)	–	–	–	–	–	–
28 Jan 2003	224[a]	10	10	10	7	7
26 Feb 2003	253	11	9	10	3	3
24 Mar 2003	279[a]	10	10	10	–	–
08 Apr 2003	294	10	8	10	–	–
25 Apr 2003	311	10	10	10	–	–
21 May 2003	337[a]	10	10	10	–	–
24 Jun 2003	371	10	10	10	–	–
11 Jul 2003	388[a]	10	0	10	–	–
(Aug 2003)	–	–	–	–	–	–
18 Sep 2003	457[a]	10	9	10	–	–
(Oct 2003)	–	–	–	–	–	–
20 Nov 2003	520[a]	10	10	10	–	6
(Dec 2003)	–	–	–	–	–	–
8 Jan 2004	569[a]	33	12	33	–	31
Sum		195	127	193	34	47

[a]All fish (Table 1; in addition to those remove from tanks and sacrificed) were measured for length and weight on this day.
[b]Histology included estimation of prevalence for oocyte stages.

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TABLE 3. Step-by-step procedure used when estimating oocyte numbers by the grid method.

Step	Overall approach	Procedure
1	Fish sampling	Carefully excise the ovary.
2	Scherle's method (Scherle 1970)	Estimate ovary volume (V_{ovary}; nearest 0.01 cm^3) from physiological seawater weight displacement of ovary ($W_{displaced\ ovary}$; nearest 0.01 g) and specific gravity of this water (ρ; nearest 0.001 g/cm^3): $V_{ovary} = W_{displaced\ ovary}/\rho$.
3	Fixation in Bouin's fluid	Preserve pieces of ovarian tissue according to Bancroft and Stevens (1996).
4	Fixation in buffered formaldehyde	Preserve pieces of ovarian tissue according to Bancroft and Stevens (1996).
5	Histology	Produce series of sections spaced sufficiently apart (to avoid considering the same oocytes more than once) by traditional methodology.
6	Image analysis: line tools	Measure the respective oocytes ($n = 10$) sectioned through the nucleus.
7	Spreadsheet	Calculate the average fresh oocyte diameter ($OD_{fresh,\ average}$; nearest 1 μm) from the relevant average sectioned diameter (equations 1 and 2).
8	Spreadsheet	Calculate the average fresh oocyte volume ($V_{oocyte,\ average}$; cm^3): $V_{oocyte,\ average} = (\pi/6)\times(OD_{fresh,\ average})^3$.
9	Image analysis: grid	Use a grid (644 points) to count the hits by oocyte phase or stage and any negative hits outside the tissue. Analyze three frames (0.004 cm^2) per fish.
10	Delesse's principle (Delesse 1847)	Calculate the area fraction of each oocyte phase or stage, as the number of hits/(644—negative hits). Set area fraction equal to volume fraction (VF)
11	Spreadsheet	Calculate the number of oocytes in each phase or stage: $(VF \times V_{ovary})/V_{oocyte,\ average}$.
12	Spreadsheet	Calculate the fecundity (F) by adding together the number of oocytes in relevant phases or stages.

Horsens, Denmark) with 17% fat, 58% protein, and a total energy content of 22.0 MJ/kg. Fish were fed a moderate ration (about 0.25% dry feed·g of $W_{body}{}^{-1}\cdot d^{-1}$; Kjesbu et al. 1991) but were initially fed an ad libitum ration to optimize acclimation to tank conditions (Table 1). In agreement with earlier information (Fordham and Trippel 1999), the appetites of the fish declined around the time of spawning (Table 1).

The water temperature in each tank was measured once per week with an electronic thermometer (calibrated before use with an oceanographic thermometer) by filling a 10-L bucket just below the surface. The temperature stratification within the tank was negligible ($\leq 0.2^\circ C$).

Collection of Ovarian samples

In addition to the aforementioned repeated measurements on all live fish, 5 females/tank were sacrificed each month, although some adjustments were made to this sampling scheme as follows (Table 2). No samples were taken in December 2002, August 2003, October 2003, or December 2003 (sufficient information on oocyte growth was considered to exist from interpolations), whereas two samples (early and late) were taken in April 2003 to better track changes associated with spawning. Furthermore, the final samples taken in January 2004 contained more than five females (tank A: $n = 6$ females; tank B: $n = 27$ females) to strengthen statistical analyses. On each sampling occasion, fish were removed one at a time, sedated, killed by a sharp blow to the head, identified by tag number, and sexed by dissection. Females were immediately processed, whereas males were ignored. Close to or during the spawning season, this routine was somewhat different; if milt was released when pressure was applied, the fish was returned to the tank for later identification and euthanization. At sacrifice (following the standard routine of starvation for a few days to empty the stomach), liver weight (W_{liver}), visceral weight (excluding gills), and ovary weight (W_{ovary}) (all three organs to nearest 0.01 g) were recorded along with W_{body} and L_{total}. Ovary volume (V_{ovary}) was measured by use of Scherle's (1970) method (Table 3).

Fresh Oocyte Diameter

Just after measurements of ovary size, a small subsample (≈0.5 g) was taken from the middle part of the right ovarian lobe (assuming ovarian homogeneity; Kjesbu and Holm 1994) and was placed in 4°C isotonic physiological saltwater (Kjesbu et al. 1996). The fresh oocyte diameter (OD_{fresh}) of the leading cohort (LC) was measured (nearest 1 μm) semiautomatically by modern digital technology (Thorsen and Kjesbu 2001; Table 2). The mean of 10 oocytes was presented as the LC diameter and taken as a reliable measure of the reproductive phase of each individual (West 1990; Kjesbu 1994). This whole-mount protocol was initiated on day 100 (26 September 2002; Table 2), around the time when the fish were expected to enter vitellogenesis (Kjesbu 1991) for the first time (see above). Fish with an LC diameter less than 250 μm were in the immature, regressing, or regenerating phase; those with an LC diameter between 250 and 850 μm were in the developing phase; and those with an LC diameter greater than 850 μm were in the spawning capable phase (Sivertsen 1935; Kjesbu 1991; Kjesbu et al. 1996; the complete terminology is described by Brown-Peterson et al.

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TABLE 4. Short microscopic description of the cytoplasm in different phases of primary oocyte development in Atlantic cod (revised from Shirokova 1977), and the corresponding range in diameter for each phase. The tissue was fixed in Bouin's fluid before histological processing. Oocyte diameter (OD) was obtained from samples embedded in HistoResin for the present study, whereas Shirokova (1977), used traditional paraffin wax. (– No information available).

Developmental phase	Description of cytoplasm	OD (μm), present study	OD (μm), Shirokova (1977)
1	Homogeneous cytoplasm, stains weakly.	8–46	–
2	Examples of small areas in the cytoplasm that stain more strongly.	38—80	>16
3	Small areas that stain more strongly are evenly distributed throughout the whole cytoplasm.	78–111	–
4a	A distinct circumnuclear ring (CNR) is located centrally in the cytoplasm.	106–171	73–121
4b	The CNR is partly dislocated towards the periphery of the cytoplasm, and the structure appears somewhat less distinct than in the previous phase.	130–190	91–165
4c	The CNR is located at the periphery of the cytoplasm and has a patchy appearance.	141–190	139–190

2011, this special issue). The time of initiation of vitellogenesis was related to the autumnal equinox (23 September 2002 and 2003; days 97 and 462, respectively; Kjesbu et al. 2010c).

Histology

For each fish, two ovarian samples were obtained (Table 2); one sample was fixed in 3.6% phosphate-buffered formaldehyde (≈0.5–3.0 g), and the other sample was fixed in Bouin's fluid (≈0.02–0.15 g; Bancroft and Stevens 1996). Fixed samples were embedded in methyl methacrylate (HistoResin, Heraeus Kulzer, Germany), sectioned (4 μm), and stained with 2% toluidine blue and 1% sodium tetraborate. The formaldehyde-fixed tissue sections were used to get a first overview of the different cell types present in the ovary (by studying relatively large histological sections) and to calculate the number of oocytes (see below), whereas the Bouin's fluid-fixed tissue sections were used to conduct highly magnified examination of cytoplasmic structures (Sorokin 1957; Tomkiewicz et al. 2003) in the smallest cells present (by studying relatively small histological sections) and to perform the associated numerical calculations of primary growth oocytes (see below).

Oocyte Classification

In addition to standard classification schemes including oogonia (OG), PVOs, CAOs, early vitellogenic oocytes (EVOs), late vitellogenic oocytes (LVOs), and hydrated oocytes, the PVO stage was further subdivided into different phases (1, 2, 3, 4a, 4b, and 4c) by adopting the terminology of Shirokova (1977). In contrast to Shirokova (1977), phase 4a in the present study was characterized by a distinct CNR instead of an indistinct CNR (due to differences in histological protocols; Table 4). Also, we prefer to use the term "CNR" following Gerbilskii (1939; see also Sorokin 1957) instead of the term "peripheral ring." Because the distinction between phases 4b and 4c was not always clear, these two phases were combined into "phase 4bc" during estimation of oocyte numbers (see below). The range in oocyte diameter (OD) for each phase was tabled and contrasted with the data of Shirokova (1977; Table 4). The EVOs showed yolk granules in the periphery of the cytoplasm, while in LVOs these were spread throughout the cytoplasm. The hydrated oocytes and POFs (Saborido-Rey and Junquera 1998; Skjæraasen et al. 2009; Witthames et al. 2010) were used as spawning markers. However, due to the most recent documentation of the long life span of POFs in Atlantic cod ovaries (Witthames et al. 2010), only hydrated oocytes were used to delimit the spawning season.

Oocyte Quantification and Associated Definitions

Relative proportions. The prevalences (%) of the different phases of the PVO stage (phases 4a, 4b, and 4c), the subsequent oocyte stages (CAO, EVO, LVO, and hydrated oocyte), and POFs were estimated for all Bouin's fluid-fixed ovaries (Table 2). Here, adopting the traditional definition of prevalence as a binary term used to indicate the presence or absence of a structure in the analyzed visual field, prevalence was calculated as the sum of individuals with the defined criterion divided by the total number of individuals in the sample. Note that some slides contained few examples of a given structure but were still scored. Oogonia and PVO phases 1, 2, and 3 were also examined, but no data are presented because there were indications of underscoring of these tiny cells, especially when large, swelling oocytes dominated in the sample. This risk of visually overlooking small structures under the microscope also applied to POFs, but because of their importance in documenting actual spawning, all available sections were carefully reexamined, searching in particular for these structures.

Number estimation by the grid method. A random subset of females in their first maturity cycle (Table 2) was used for quantification of oocytes by a technique we developed, called

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the "grid method" (Table 3). Specifically, this method included the following key components:

1. Assessment of the fresh V_{ovary} by use of Scherle's (1970) method
2. Prediction of the average fresh volume of oocytes in different PVO phases (4a, 4b, and 4c) and in subsequent stages (CAO, EVO, and LVO) from diameter measurement of sectioned oocytes
3. Measurement of the ovarian volume fraction of these oocytes by using Delesse's (1847) principle
4. Calculation of oocyte numbers from simple packing theory of spheres
5. Summation of oocyte numbers.

The last component was analogous to the estimation of total F, which was used in the calculation of relative somatic fecundity (RF_S; determined as $F/[W_{body} - W_{ovary}]$) and OPD (calculated as F/W_{ovary}).

All scoring of oocyte phases or stages and the collection of information on OD and ovarian volume fraction (hits were marked with different colors depending on the cell-type category chosen) were undertaken on histological slides. However, due to component 1 above, it was necessary to back-calculate all sectioned diameters to fresh values. The relationship between OD (PVOs and CAOs) as measured in Bouin's fluid-fixed tissue sections (OD_{Bouin}; nearest 1 μm) and OD_{fresh} (nearest 1 μm) was as follows:

$$OD_{fresh} = (0.988 \times OD_{Bouin}) + 19 \quad (1)$$

(adjusted $r^2 = 0.927$, df = 6, $P < 0.001$). The relationship between OD (PVOs, CAOs, EVOs, and LVOs) measured from formaldehyde (formalin) fixed tissue sections ($OD_{formalin}$; nearest 1 μm) and OD_{fresh} was

$$OD_{fresh} = (1.110 \times OD_{formalin}) - 19 \quad (2)$$

($r^2 = 0.996$, df = 15, $P < 0.001$). Individual OD was calculated as the mean of the short and long axes. In histology, only oocytes that were sectioned through the nucleus were considered. Care was taken that the same type of oocyte was contrasted by consulting the respective LC diameter. Generally, OD_{fresh} was about 7% larger than OD_{Bouin} and $OD_{formalin}$.

Number estimation by the autodiametric method. Prior to the first (day 224) and second (days 520 and 569) spawning seasons, the standing (potential) F (CAOs, EVOs, and LVOs) was estimated by the autodiametric method (Thorsen and Kjesbu 2001; Table 2). Additional specimens not yet spawning on day 253 were also included (Table 2). Mean diameter found automatically in whole mounts (wm; $OD_{formalin,wm,mean}$; nearest 1 μm) from 200 developing oocytes (>250 μm) was entered into equation (3) from Thorsen and Kjesbu (2001) to obtain OPD:

$$OPD = (2.139 \times 10^{11}) \times (OD_{formalin,wm,mean})^{-2.700} \quad (3)$$

($r^2 = 0.988$, df = 45). The OPD results from the 10 spawning capable (vitellogenic) individuals sampled on days 224 and 253 were directly compared with the similar data from the grid method. Here, the autodiametric method was assumed to give fully realistic OPDs for $OD_{formalin,wm,mean}$ values of 300 μm and greater (see operational limitations for the smaller, transparent oocytes as described by Thorsen and Kjesbu 2001). The same formaldehyde fixative as above was used, and the following relationship (Svåsand et al. 1996) was identified between fixed OD ($OD_{formalin,wm}$; nearest 1 μm) and OD_{fresh}:

$$OD_{fresh} = (0.947 \times OD_{formalin,wm}) + 19 \quad (4)$$

(425 μm < $OD_{formalin,wm}$ < 675 μm; $r^2 = 0.951$, df = 8, $P < 0.001$). Thus, an individual Atlantic cod oocyte swells by about 1–2% when put into this fixative. Equation (4), along with equations (1) and (2), was used in calculation of OD_{fresh} for the LC oocytes (i.e., in standardization exercises for proper method comparisons).

RESULTS

Tank Conditions and General Fish Performance

Reproductive information from the two tanks was pooled together as there was no evidence of any difference in fish husbandry conditions and the resulting oocyte production. Measured water temperature ranged between 7°C and 10°C, following the normal seasonal pattern seen in north temperate waters. The fish in the two tanks were maintained under similar temperatures (Wilcoxon's signed rank test: $P = 0.859$; $n = 67$ observations/tank); mean temperature was 9.04°C (SD = 0.65°C) in tank A and 9.03°C (SD = 0.64°C) in tank B. The feeding rations also appeared to be similar over time (analysis of covariance [ANCOVA], slope: df = 14, $P = 0.823$; intercept: df = 15, $P = 0.798$). Likewise, the RF_S as standardized by maturity stage (LC diameter) along the x-axis was not significantly different (days 520 and 569; ANCOVA, slope: df = 33, $P = 0.263$; intercept: df = 34, $P = 0.259$).

During the 569 d of the experiment, the females grew from an average of 497 g (SD = 32 g; $n = 11$) to 3,130 g (SD = 641 g; $n = 33$). They were generally in excellent body condition (Fulton's condition factor [$K = 100 \times \{W_{body}/L_{total}^3\}$] fluctuated around 1.1–1.2; data not shown). A few females were immature at age 2 (day 224), and one female was still immature in the next spawning season (day 569) as evidenced from whole mounts (Figure 1) and supported by histology (see below). The experiment provided access to all five reproductive phases (i.e., immature, developing, spawning capable, regressing, and regenerating; Figure 1). The subsequent analysis focuses primarily on the two first phases.

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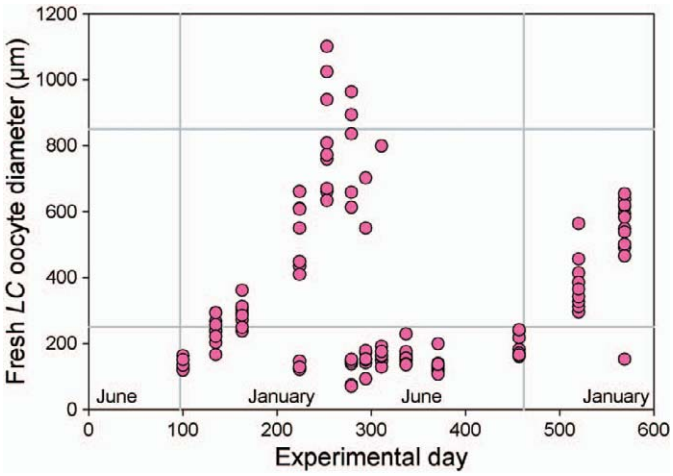


FIGURE 1. Fresh leading cohort (LC) oocyte diameter in Atlantic cod as measured throughout the 569-d experiment. Vertical lines refer to the time of the autumnal equinox. The lower horizontal line separates immature or regressing individuals (following the first spawning season; <250 μm) from developing individuals (250–850 μm); the upper horizontal line indicates initiation of oocyte maturation and thereby spawning (>850 μm).

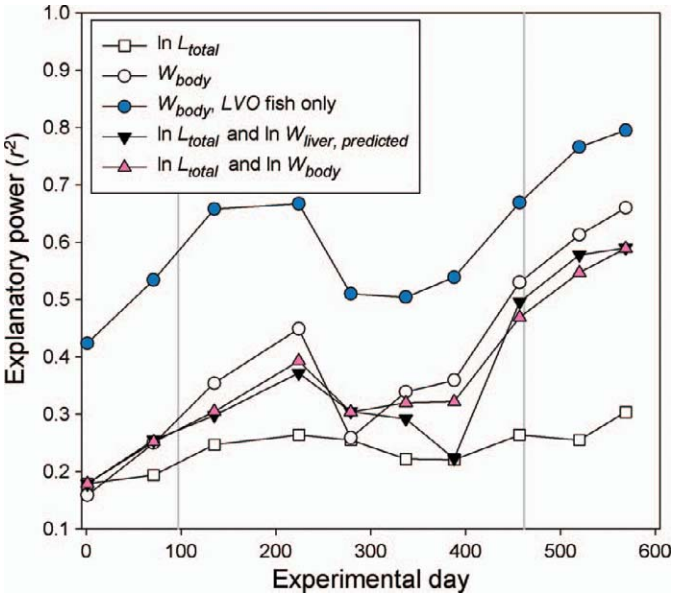


FIGURE 2. The explanatory power (r^2) of various fecundity (F) models for Atlantic cod over time. The number of cortical alveolar oocytes, early vitellogenic oocytes, and late vitellogenic oocytes (LVO) found by the autodiametric method in prespawning females ($n = 31$) at the end of the experiment (day 569; Table 2) was set as F and related to the following combination of explanatory variables: total length (L_{total}), whole-body weight (W_{body}), L_{total} and predicted liver weight ($W_{liver,predicted}$), and L_{total} and W_{body} (ln = natural logarithm transformed data). Note that L_{total} and W_{body} were measured at different times during the course of the experiment from live fish, while F was measured only once (i.e., when those same fish were sacrificed). The $W_{liver,predicted}$ in live fish was obtained by use of multiple regressions established from sacrificed fish (see Figure 3). For W_{body}, the test was further restricted to LVO females only ($n = 22$; for which the mean oocyte diameter in formaldehyde-fixed sections was > 400 μm). The first spawning season extended approximately from day 250 to day 300 (see the appearance of hydrated oocytes as the spawning marker in Figure 7).

Influence of Body and Liver Size on Final Fecundity as Determined by the Autodiametric Method

Overall, W_{body} was the best predictor of F (the number of CAOs, EVOs, and LVOs) on day 569, especially when limiting the analysis to LVO females to account for downregulation (see Discussion), as reflected in an r^2 close to 0.80 (Figure 2). About 65% of this variation could be explained by W_{body} data collected many months earlier from the same fish (day 135–224; see Table 2; Figure 2). However, a comparison between W_{body} ($n = 22$) on days 224 and 569 showed a very close relationship ($r^2 = 0.816$; $P < 0.001$). In contrast to this situation, L_{total} as a single predictor explained only up to about 30% of the variation in F, although the regressions linearized by logarithmic transformation were still significant ($0.001 < P < 0.026$; Figure 2).

Generally, body metrics measured on live fish during the spawning season and the subsequent regressing and regenerating periods (≈days 250–400) had less influence on subsequent F than metrics measured during the developing period (i.e., after the autumnal equinox until spawning; ≈days 100–250 and 400–600; Figure 2). The length of the various maturity periods is detailed below. A model that included predicted W_{liver} ($W_{liver,predicted}$) based on sacrificed fish (Table 2; Figure 2) as an index of condition together with L_{total} did not explain more variation in F than a model that included L_{total} and W_{body} (Figure 2); sometimes the model with L_{total} and $W_{liver,predicted}$ was better, and sometimes the model with L_{total} and W_{body} was better. However, the analysis of $W_{liver,predicted}$ as a linear function of L_{total} and W_{body} (following tests on a range of statistical options and combinations) gave some insight into the temporal influences on $W_{liver,predicted}$ (Figure 3). The statistical effect of measured L_{total} in the multiple regression disappeared in the late spawning season and in the subsequent regressing period (days 279–337; $P \geq 0.367$) and also when the fish were approaching spawning for the second time (day 520; $P = 0.242$). Furthermore, $W_{liver,predicted}$ could not be effectively given ($P = 0.224$) during peak spawning (day 253) and in the assumed regenerating period (days 371–388; $P \geq 0.054$; see below). Taken together, the results provided clear evidence that the event of first spawning subsequently introduced a high level of noise in the liver data compared with the earlier situation characterized by high predictability (Figure 3).

Validation of the Grid Method

The grid method gave generally 16.6% lower OPD values than the autodiametric method for oocytes that were classified as CAOs, EVOs, and LVOs and represented by their LC diameters (ANCOVA, slope: df = 16, $P = 0.395$; intercept: df = 17, $P = 0.016$; Table 2, days 224 and 253; Figure 4). There was a clear negative trend in the ratio between the two OPD data sets as a function of LC diameter (adjusted $r^2 = 0.822$, $P < 0.001$). Analysis of sectioned versus whole-mount oocytes showed that the diameter of the larger sectioned oocytes was biased upwards, causing the grid method to consistently

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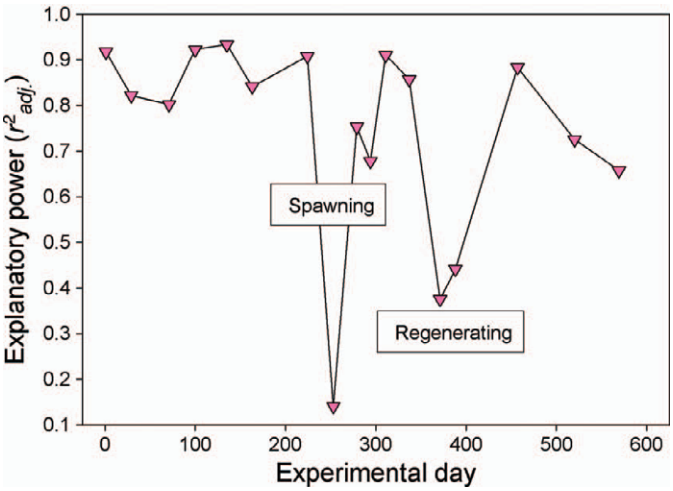


FIGURE 3. Time series estimates of the explanatory power (adjusted r^2) of the multiple regression between Atlantic cod liver weight as the dependent variable and total length and whole-body weight as the independent variables. Spawning and regenerating periods are indicated. Number of females sacrificed for the last sampling point was 33; sample size was 10 females at all other points.

underestimate OPD. One possible explanation for this phenomenon appeared to be a much greater range in oocyte size for larger oocytes than for smaller oocytes (Figure 5). Consequently, the following correction factor (CF_OD_{fresh}) was established after calibration:

$$CF_OD_{fresh} = [10.91 \times e^{(-0.012 \times ODfresh)}] + 0.87 \qquad (5)$$

($OD_{fresh} > 350$ μm; adjusted $r^2 = 0.924$, df = 7, $P < 0.001$; 32 iterations), where OD_{fresh} is that recalculated from $OD_{formalin}$

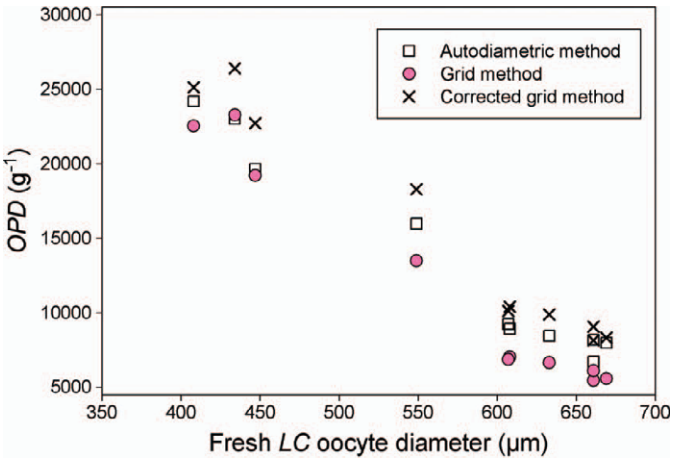


FIGURE 4. Oocyte packing density (OPD; number of oocytes/g of ovary) for developing oocytes of Atlantic cod in relation to fresh leading cohort (LC) oocyte diameter estimated by the grid method (Table 3), the corrected grid method (see Results), and the autodiametric method. Samples with LC diameters less than 500 μm contained developing oocytes characterized as cortical alveolar, early vitellogenic, and late vitellogenic oocytes, while for LC diameters greater than 500 μm the only developing type was late vitellogenic oocytes.

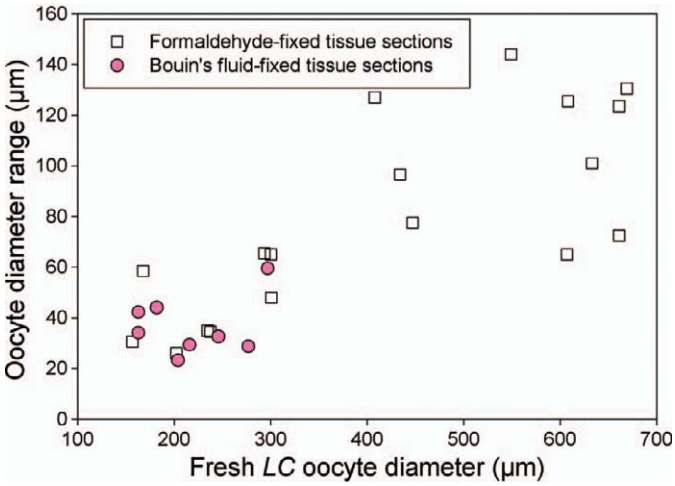


FIGURE 5. Range (maximum value − minimum value) in diameter of various sectioned Atlantic cod oocytes (previtellogenic oocytes to late vitellogenic oocytes) fixed either in formaldehyde or Bouin's fluid plotted versus the corresponding fresh leading cohort (LC) oocyte diameter.

(equation 2). Consequently, for OD_{fresh} values of 350–400 μm, the CF_OD_{fresh} is around 1, while for OD_{fresh} values of 600–650 μm the CF_OD_{fresh} is approximately 0.87. After use of equation (5), the previous situation was reversed, resulting in a generally 12.8% higher OPD from the corrected grid method (ANCOVA, slope: df = 16, $P = 0.353$; intercept: df = 17, $P = 0.020$), but differences became negligible for the largest oocytes (Figure 4).

Characterization of Oocytes and Postovulatory Follicles

The illustrations by Shirokova (1977), which represent the different PVO phases (1, 2, 3, 4a, 4b, and 4c) and CAO and were reproduced by hand from histological sections of Baltic Atlantic cod, detail very much the same morphological information as in the present photomicrographs (Figure 6). Shirokova's (1977) reported diameters for phases 4a and 4b were in the low range compared with our results, but the diameters fully overlapped for phase 4c (Table 4). Representative examples of EVOs and POFs are also given in Figure 6.

Presence of Primary and Secondary Oocytes

The various types of oocytes showed large fluctuations in prevalence (Figure 7). This included successive "waves" of progressing stages. An exception to this was OG and PVO phases 1, 2, and 3, which apparently were present at all times (i.e., we considered the decline in prevalence during spawning for these very small cells to be an observational artifact; data not shown). The observation that OG tended to be less frequent in females developing for the second time was not pursued further. Importantly, phases 4a, 4b, and 4c were not present in immature fish (days 0 and 29) but appeared with full strength one after the other around the time of the autumnal equinox, followed by the sequential production of CAOs, EVOs, LVOs, hydrated oocytes, and POFs (Figure 7). After the first spawning season, the prevalence of phases 4b and 4c was noticed to build up gradually

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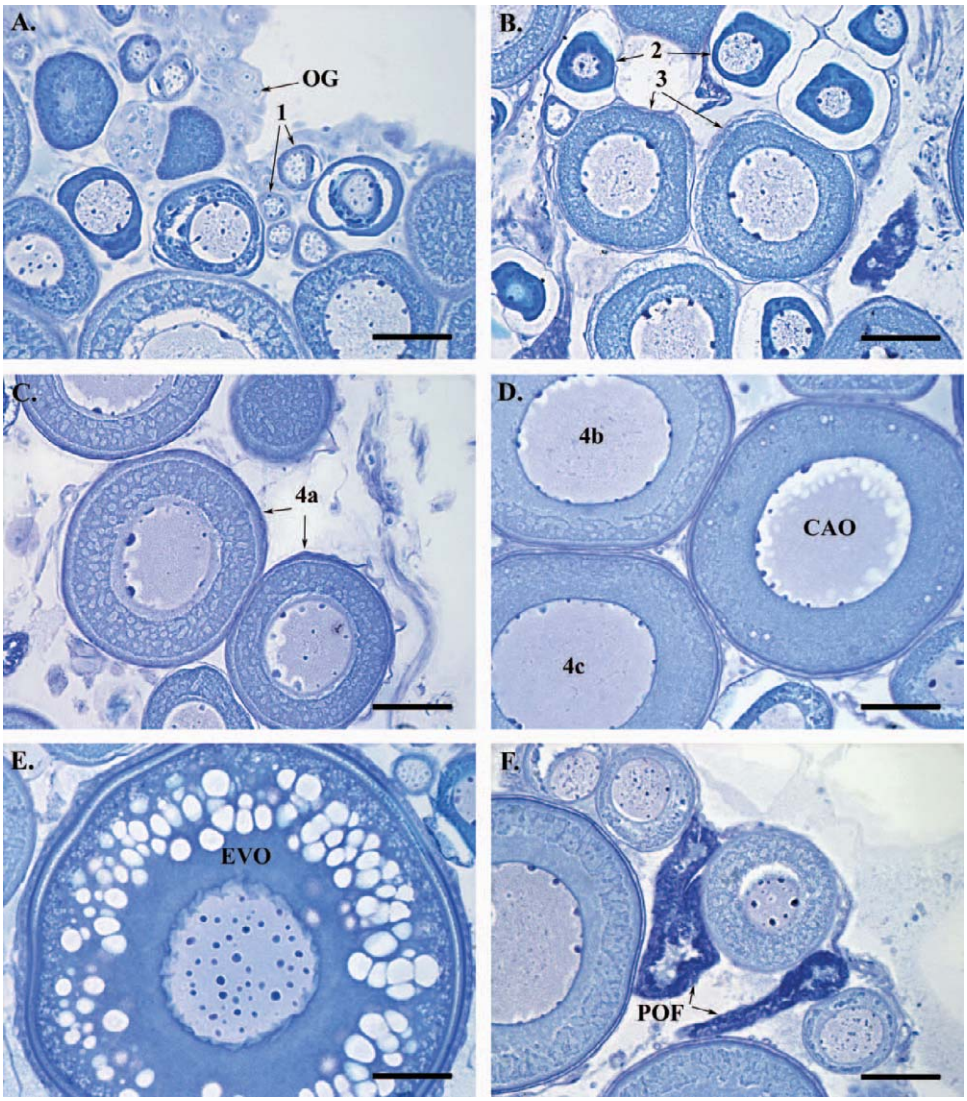


FIGURE 6. Histological appearance of various Bouin's fluid-fixed oocytes as observed under the light microscope for Norwegian coastal Atlantic cod after methyl methacrylate embedding and toluidine blue staining. The different previtellogenic oocyte (PVO) phases (1, 2, 3, 4a, 4b, and 4c) follow those of Shirokova (1977). Specific criteria for classification of these phases are given in Table 4. Cell types and structures (scale bar = 50 μm) are **(A)** oogonium (OG) and PVO phase 1; **(B)** PVO phases 2 and 3; **(C)** PVO phase 4a; **(D)** PVO phases 4b and 4c and a cortical alveolar oocyte (CAO); **(E)** early vitellogenic oocyte (EVO); and **(F)** postovulatory follicle (POF).

over time instead of increasing abruptly (i.e., as occurred before the first spawning season), but again the value peaked around the autumnal equinox, followed by the similar cyclic production of developing oocytes (up to the second spawning season). Phase 4a apparently formed a standing stock of oocytes after sexual maturity, while virtually all phase 4b and 4c oocytes were transformed into subsequent developmental stages. The few mentioned immature fish at ages 2 and 3 showed oocytes in phase 4a or 4b. Postovulatory follicles from the first spawning season were still seen on day 569 (i.e., after approximately 300 d or less, although they were then extremely small and required high magnification to be spotted with a reasonable level of certainty).

Numbers of Primary and Secondary Oocytes

The numerical production of primary and secondary oocytes was standardized either by ovary size or by ovarian-free body size via estimation of OPD (Figure 8; grid method estimates) and RF_S (Figure 9; grid and autodiametric method estimates), respectively.

The minimum oocyte size studied was around 100 μm, probably explaining why PVO phase 4a (Table 4), as opposed to the other oocyte types considered, is not represented with a baseline OPD of 0 in Figure 8. As expected, all panels show indications of a decline in OPD with LC diameter. Roughly speaking, the maximum OPD value of phase 4a was twice the value for phase 4bc or CAOs, five times the value for EVOs, and 10 times the

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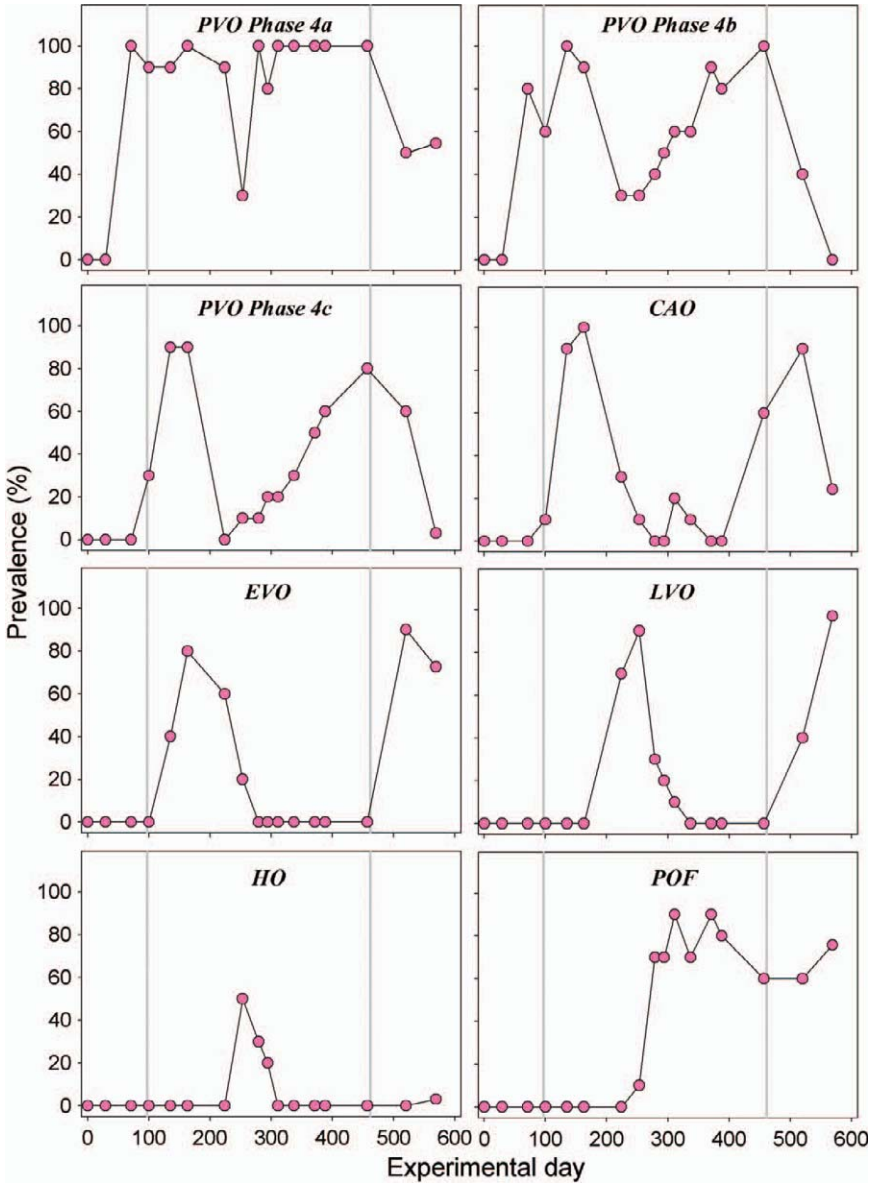


FIGURE 7. Prevalence of the different previtellogenic oocyte (PVO) phases (4a, 4b, and 4c), subsequent stages (cortical alveolar oocyte [CAO], early vitellogenic oocyte [EVO], late vitellogenic oocyte [LVO], and hydrated oocyte [HO]), and postovulatory follicles (POF) examined in Atlantic cod during the experiment (Bouin's fluid-fixed samples). The autumnal equinox for each year is shown (gray vertical lines). Between days 0 and 520, 9–11 females (normally 10) were analyzed on each sample date; 33 females were analyzed on the final sample date (day 569; Table 2).

value for LVOs. Concurrently, there was a large increase in ovary size (data not shown).

The RF_S (CAOs, EVOs, and LVOs) started off with an early production of CAOs already around 180 μm, followed successively by the production of EVOs and LVOs but then leveling off (Figure 9). There were indications of a decline in RF_S at the largest LC diameters considered. Hence, overall the RF_S showed indications of a dome-shaped curve. At the defined plateau (LC diameter > 350 μm), RF_S was 18% higher for second-time spawners (mean = 1,058 oocytes/g of fish) than for first-time spawners (mean = 897 oocytes/g of fish; ANCOVA, slope: df = 42, $P = 0.369$; intercept: df = 43, $P = 0.035$).

DISCUSSION

As expected, the corrected grid method gave very much the same OPD results as the autodiametric method in the upper diameter range but produced higher values in the lower diameter range of secondary growth oocytes (from CAO to LVO). It is reasonable to assume that histological screening will pick up all or nearly all developing oocytes, whereas automated analyses

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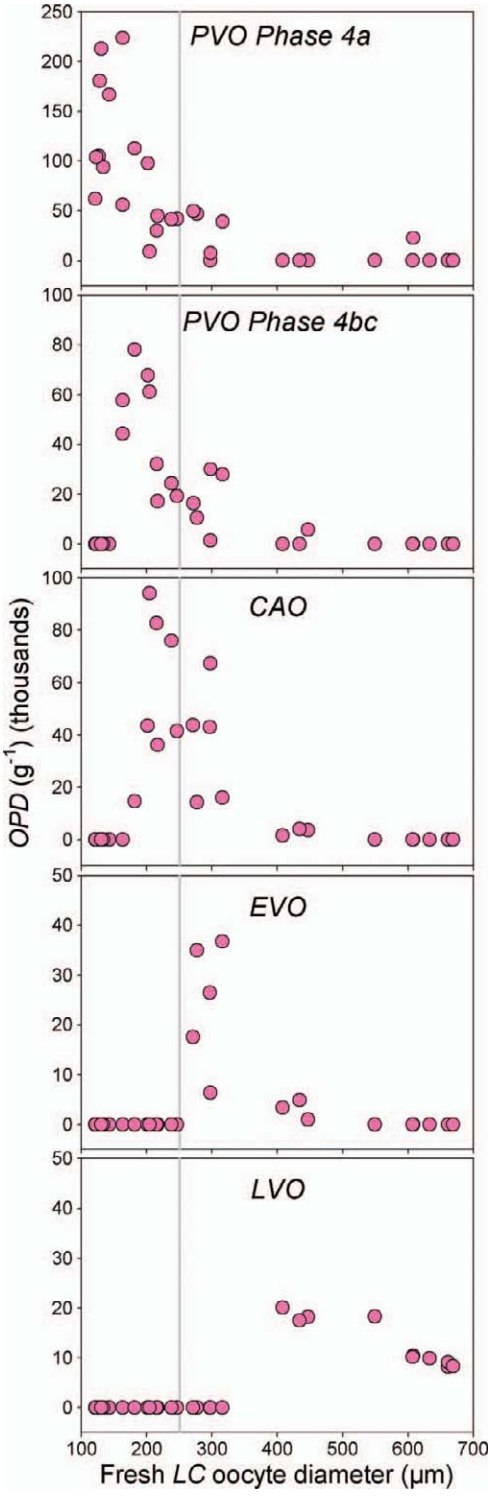


FIGURE 8. Oocyte packing density (OPD; number of oocytes/g of ovary) of the various Atlantic cod oocyte types examined with the grid method (previtellogenic oocyte [PVO] phase 4a; PVO phases 4b and 4c combined [phase 4bc]; cortical alveolar oocyte [CAO]; early vitellogenic oocyte [EVO]; and late vitellogenic oocyte [LVO]) in relation to fresh leading cohort (LC) oocyte diameter. The line refers to the common threshold oocyte diameter (250 µm) used to separate between immature and developing individuals. Note the different *y*-axis ranges.

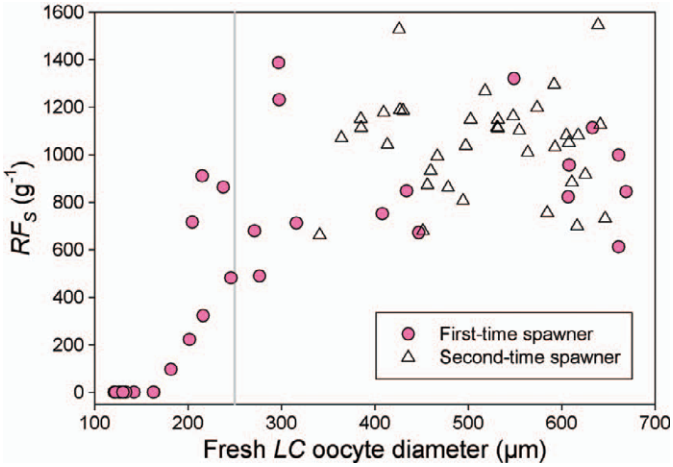


FIGURE 9. Relative somatic potential fecundity (RF_S; number of cortical alveolar, early vitellogenic, and late vitellogenic oocytes per gram of ovary-free body weight) of Atlantic cod as estimated by the grid method ($n = 34$; see Table 2 for sample dates) and autodiametric method ($n = 37$; Table 2, days 520 and 569 only) as a function of fresh leading cohort (LC) oocyte diameter. Line marks the common threshold oocyte diameter (250 µm) used to separate immature and developing reproductive phases.

on whole mounts will overlook some of the more transparent oocytes that are actually developing. Overall, the corrected grid method showed about 13% higher estimates than the autodiametric method, which is a sensible result when also taking into account the various levels of uncertainty (see below). Thus, we also believe that the present OPD values for primary oocytes are realistic approximations.

The grid method should be considered a practical, user-friendly alternative to already existing concepts, theories, and models. Actually, it contains some similarities with the so-called Weibel method (Weibel and Gomez 1962; Emerson et al. 1990; estimation of volume fractions) but mainly is a "light" version of the advanced OPD theory method that was specially developed for studies of fish oocytes (Kurita and Kjesbu 2009). Today, the Weibel method is largely outcompeted by the disector method because the Weibel method requires assumptions of particle shape and particle size distribution (presently ignored), whereas the disector method is free of assumptions (but requires the operational rules to be followed strictly). However, this does not necessarily imply that one model gives better results than the other. As an example, both Greer Walker et al. (1994) and Korta et al. (2010) indicated that there were about 400,000 primary oocytes/g of ovary when the OD was approximately 100 µm despite their use of the Weibel method on Atlantic mackerel *Scomber scombrus* (Greer Walker et al. 1994) and the advanced OPD theory method on European hake *Merluccius merluccius* (Korta et al. 2010). For Atlantic mackerel, the ovary weight data were obtained elsewhere (P. Witthames, Center for Environment, Fisheries, and Aquaculture Science, Lowestoft, UK, personal communication). At an OD of 150 µm, European hake (Korta et al. 2010) and Atlantic cod show about 200,000

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primary oocytes/g of ovary. Altogether, these results support the view that the bearing principle relates to the "closest packing density of spheres" used in physics. Also, the examples clarify that primary OPD figures are highly sensitive to small changes in diameter. Recently, Newman et al. (2007) also included primary oocytes in their study of ovarian maturation in Murray cod *Maccullochella peeli* by use of the Weibel method, but unfortunately they did not show any OPD data. In relation to the universal OPD formula (Kurita and Kjesbu 2009), we ignored particle shape (i.e., the k factor) and specific gravity of the ovary (i.e., ρ_o) and we replaced volume-based mean OD with arithmetic mean OD. The latter would have introduced a significant error in case of broad oocyte size distributions, as was seen in olive flounder *Paralichthys olivaceus* (Kurita and Kjesbu 2009), but here the oocyte classes were studied separately, thus eliminating this problem (Korta et al. 2010). Any variation in ρ_o should also be unimportant (Kurita and Kjesbu 2009). However, the fact that we defined the Atlantic cod oocyte as spherical while it is actually ellipsoid (Thorsen and Kjesbu 2001) might be an issue to consider. The new equation (5) is clearly important for correcting biased oocyte measurements from sections. This equation should be further improved by including more data points and by extending the analyses to lower oocyte sizes, although it is sufficient for the present analysis. The likely explanation is that the actual measurement of each oocyte was satisfactory but the selection of oocytes was biased. Here, the height of the nucleus normal to the section plane is central, as only oocytes sectioned through the nucleus were considered (see Andersen 2003). Consequently, Greer Walker et al. (1994) introduced a correction factor based on relative nucleus sizes, and since we used relative oocyte sizes our approach may seem mistaken at first glance. However, we circumvented the problem by calibrating the histological oocyte measurements with whole-mount data. In future studies, the grid method can probably be simplified further by using one instead of two fixatives after conducting some pilot examinations of stage-specific oocyte size. The logical candidate for exclusion is Bouin's fluid, which contains picric acid and therefore can become explosive if allowed to dry out. However, this fixative appears particularly suitable for studies of CNR, explaining why it was presently included along with the traditional buffered formalin; the PVO phase classification scheme ought to be reliable and in line with the reference work of Shirokova (1977), who obviously fixed the oocytes in the same way.

To our knowledge, the present experiment is the first study of Atlantic cod wherein the different types of oocytes have been assessed according to stereological principles. The established overall model of F shows that RF_S can be said to follow a dome-shaped curve over time. In this study, RF_S already started to build up at 180 μm, when CAOs recruited to F. This is surprisingly early as this threshold value is commonly set at 250 μm (Sivertsen 1935; Kjesbu 1991), perhaps because such detailed examinations have not been undertaken before. At the other end of oocyte growth, RF_S apparently starts to drop at an LC diameter of approximately 550 μm, which largely agrees with Skjæraasen et al. (2010), who stated that the F of Atlantic cod in the northeast Arctic peaked at an LC diameter of 614 μm based on statistical analysis of extensive field data. Thus, in Atlantic cod as in many other fish species, F is downregulated prior to spawning (Kurita et al. 2003; Thorsen et al. 2006; Kennedy et al. 2008, 2009; Kjesbu 2009). This 19-month tracking study on Atlantic cod in good condition clearly showed that the final F was closely related to the body growth history (as all individuals were born at the same time, their body size directly reflects their growth rate). Not surprisingly, body characteristics during the period of vitellogenesis appeared to have the strongest impact on F, but interestingly W_{liver} was much easier to predict before the first spawning season than afterward. The most obvious reason is that varying reproductive investment puts a varying drain on the body resources (Stearns 1992).

The experimental setup gave unique insight into the fate of primary growth oocytes. The main finding was that the Atlantic cod oocytes recruit to the developing mode at around the time of the autumnal equinox. This phenomenon has just been discovered and was most important for the successful establishment of a maturity formula used to predict spawning time in Atlantic cod at different temperatures (Kjesbu et al. 2010c). However, the underlying primary growth process was not considered. Here, we document that the growth of previtellogenic oocytes seems to be remarkably steered by day length, which is basically a new field of research, at least for marine fish. The fact that phase 4a PVOs could be as small as 100 μm implies that the starting point for the developing phase is far below 250 μm. However, for practical purposes, the 250-μm threshold value still seems appropriate to allow reasonable certainty that the fish is actually developing, provided that fish condition is not too low (Skjæraasen et al. 2009). Note that some immature fish in the present study apparently moved to phases 4a and 4b but then stopped progressing further. Furthermore, the fact that we pooled phases 4b and 4c in the quantitative analysis to avoid problems with interpretation points in the same direction. Nevertheless, we agree with the statements of Shirokova (1977) and Holdway and Beamish (1985) that the fish in phase 4c can be said to be sexually mature.

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and the defined deliverables of FRESH Working Group 2 and the Northwest Atlantic Fisheries Organization Working Group on Reproductive Potential (Edward A. Trippel, coordinator). We specially thank the organizers of the Fourth Workshop on Gonadal Histology of Fishes for selecting this publication as a candidate for their special issue, and we are grateful to the three reviewers for their most valuable efforts. In the experiment, animal welfare conventions and guidelines were strictly followed. Vemund Mangerud is deeply thanked for his careful fish husbandry.

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