



## Reproductive strategy, egg characteristics and embryonic development of Greenland halibut (*Reinhardtius hippoglossoides*)

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Despite the commercial importance of Greenland halibut (GH), important gaps exist in our knowledge of the reproductive and early life stage biology for this species. The present study examined through laboratory experiments the spawning strategy, realized fecundity, egg characteristics, biochemical composition, and embryonic development of GH. The results confirmed the hypothesis that GH is a single-batch spawner producing large eggs, resulting in low realized fecundity. Embryonic development and hatching time are highly dependent on incubation temperature; 50% hatching occurred after 46, 30, and 24 days at 2, 4, and 6°C, respectively. Few changes in the biochemical composition of the eggs are observed during embryonic development. Newly hatched larvae are not well developed, having a large yolk sac, no pigmentation and incomplete development of the jaws. Egg specific density confirmed the mesopelagic distribution of the eggs at sea. However, important buoyancy changes occurring in the last 3–4 days before hatching indicate that larvae hatch higher in the water column. These results are important for understanding advection and dispersion processes of GH eggs and larvae and the connectivity between spawning grounds and nursery areas.

**Keywords:** egg biochemical composition, egg buoyancy, embryonic development, fecundity, *Reinhardtius hippoglossoides*, reproductive strategy.

### Introduction

Greenland halibut (*Reinhardtius hippoglossoides* Walbaum 1792) is a deepwater fish species found in the Northern hemisphere in both the Atlantic and the Pacific Oceans. It is considered to be a species with slow growth, late maturation, and low fecundity (Junquera *et al.*, 1999; Gundersen *et al.*, 2009; Kennedy *et al.*, 2009). Although many aspects of the biology of Greenland halibut have been well studied, important gaps in the knowledge of its reproductive biology still remain.

Information on spawning season, length at maturity and potential fecundity of Greenland halibut is available for some management units (stocks), although it is limited for others (Gundersen *et al.*, 1999; Gundersen *et al.*, 2001b; Morgan *et al.*, 2003; Cooper *et al.*, 2007; Gundersen *et al.*, 2009). There are also some uncertainties about the spawning characteristics as well as the accuracy of fecundity measurements, due to down-regulation by atresia, that may lead to the overestimation of realized fecundity (Kennedy *et al.*, 2009).

Many authors have concluded that Greenland halibut is a group-synchronous determinate spawner (Gundersen *et al.*, 1999; Junquera *et al.*, 1999; Gundersen *et al.* 2010; Kennedy *et al.* 2011). However, it has been suggested that the spawning pattern of Northwest Atlantic Greenland halibut may not be determinate (Rideout *et al.*, 1999). Moreover, while Greenland halibut is considered to be a determinate spawner, it is still unclear if fish spawn their eggs in a single batch or multiple batches with long or short spawning intervals (Stene *et al.*, 1999; Albert *et al.*, 2001; Murua and Saborido-Rey, 2003; Kennedy *et al.*, 2009). Finally, some irregularities in the maturation process have been observed, suggesting that individuals do not necessarily spawn on an annual basis (Morgan and Bowering, 1997; Junquera *et al.*, 2003; Kennedy *et al.*, 2011). Thus, further studies on these issues are warranted, considering the potential for important consequences for stock-recruit relationships.

Due to their spawning strategy, spawning season and/or spawning behaviour, Greenland halibut females in spawning condition (i.e. hydrated eggs) are rarely caught by fishing gears

(Stene *et al.*, 1999; Kennedy *et al.*, 2009). This results in uncertainties in the precise determination of spawning grounds, nursery areas, and egg and larval stage distribution, as well as the duration of the spawning season. Studies dealing with the sampling of Greenland halibut egg and larval stages have been based on few observations (Haug *et al.*, 1989; Stene *et al.*, 1999; Sohn *et al.*, 2010). This is especially true for the sampling of eggs where reported total egg numbers are usually low. Nevertheless, available information indicates that Greenland halibut produce large (3.9–4.7 mm in diameter) mesopelagic eggs. Early larval stages (i.e. yolk-sac larvae) are also caught in deep waters (Jensen, 1935; Bulatov, 1983), while larvae at later developmental stages are progressively found in shallower depths in the water column (Haug *et al.*, 1989; Sohn *et al.*, 2010; Ouellet *et al.*, 2011). However, little is known about embryonic development, egg development time, and the characteristics of fertilized eggs, egg composition and buoyancy. To our knowledge, the only study on Greenland halibut egg development was carried out by Stene *et al.* (1999). They managed to follow egg development for one running female caught at sea. Unfortunately, few eggs hatched and no viable larvae were obtained.

Greenland halibut in the Gulf of St Lawrence, Canada is considered to be a distinct population (DFO, 2011). Contrary to most other identified stocks, its distributional area is less extensive and fish inhabit bottom depths between 150 and 500 m. This depth distribution, as well as the concentration of fishing grounds close to land in the Gulf of St Lawrence, makes it possible to catch live specimens on daily trips and to transport them short distances to land-based tank facilities. We took advantage of this unique situation to conduct controlled laboratory experiments related to spawning strategy and reproductive characteristics in Gulf of St Lawrence Greenland halibut. The specific objectives were to determine (i) the realized fecundity, (ii) egg characteristics and changes in biochemical composition during embryonic development, and (iii) the development time of eggs in relation to temperature in Greenland halibut.

## Material and methods

### Broodstock

Greenland halibut males and females used for the experiments were caught with longlines in the Gulf of St Lawrence in October 2006 and 2007. Fishing activities were conducted at depths between 150 and 300 m in the area around Mont-Louis, Québec (49°14'N 65°44'W). Within 12 to 48 h following capture, fish were transported to the Maurice Lamontagne Institute (Fisheries and Oceans Canada, Mont-Joli, Québec) located at a distance of 200 km. Fish were kept in two 24 m<sup>3</sup> tanks (4 m × 4 m × 1.5 m depth) at temperatures between 3 and 5°C and salinities between 28 and 32 under natural photoperiod. The fish resumed feeding after an acclimation period of 2 to 4 weeks. They were then fed to satiation with capelin, *Mallotus villosus*, three times a week. Females were monitored daily and transferred to smaller 1.2 m<sup>3</sup> circular tanks on the first sign of swelling of the abdomen and then followed closely to detect ovulation. Spermiating males were also identified and isolated in smaller circular tanks. All fish used in the experiment were identified individually with passive integrated tags (PIT) inserted above the lateral line near the dorsal fin.

### Fertilization and egg incubation

In both years of the experiments, spawning began in early January. The ovulation of the females was assessed by first looking for swollen abdomen and the swelling and reddening of the genital pore. Females were then anaesthetized in a solution of metomidate hydrochloride (6 mg l<sup>-1</sup>) (Mattson and Riple, 1989) and examined for the presence of ovulated eggs by applying a gentle pressure to the sides of the fish. When females were ready to spawn, the anal region was dried and all eggs were collected by stripping. Female identification number, fork length (cm), weight (g), and total weight (g) of stripped eggs were noted. Somatic weight was determined by subtracting the weight of stripped eggs from total fish weight. Three samples (~0.5 g) of non-fertilized eggs were taken, weighed (0.0001 g) and the number of eggs counted. Total number of eggs produced was calculated from mean egg weight in the samples and total weight of stripped eggs. An egg weight index was also calculated by dividing total weight of stripped eggs by total fish weight.

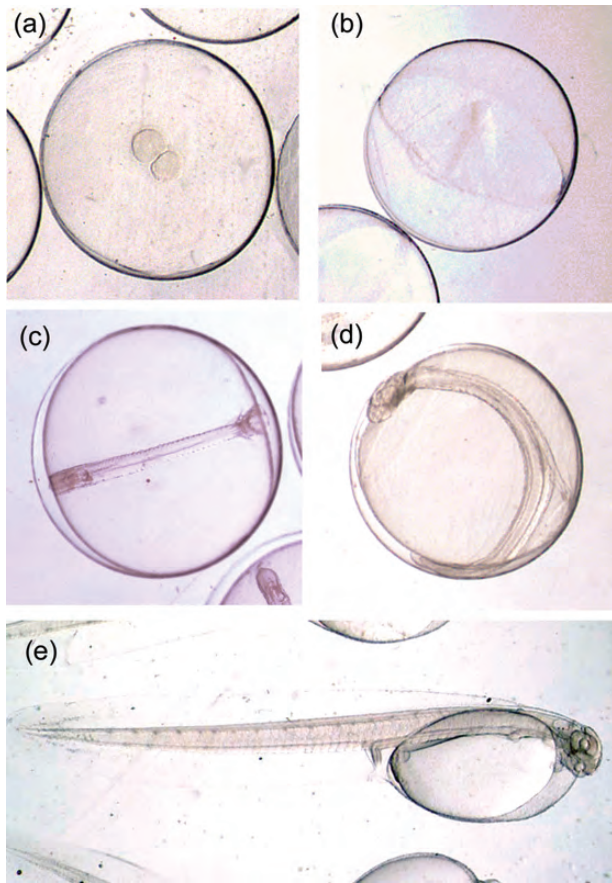
Egg fertilization was done by adding semen and filtered seawater to the eggs using a 100:1:100 ratio for egg, semen and seawater volumes. Eggs were then stored at 4°C in the dark for 10 min and transferred to plankton-Kreisel type incubation tanks (Aiken and Waddy, 1989). All manipulations were done at low light intensity in a well-oxygenated seawater bath (salinity 30–32) at 4°C. A solution of Vidalife™ (1 ml per 15 l of seawater) was also used in all manipulations as a water conditioner and coating to reduce friction and abrasion, Greenland halibut being susceptible to handling which easily removes the mucus layer and cause descaling. Following fertilization, adult fish were transferred to recovery tanks and monitored daily for a period of two weeks to detect further ovulation and spawning events.

In the winter of 2007, all fertilized eggs of each female were incubated at one of the randomly selected temperatures (2, 4, or 6°C), while in the winter of 2008, egg incubations were done at temperatures between 4 and 5°C only. For both years, salinity was maintained at a fixed level of 32 during the whole incubation period. Fertilized eggs were sampled every two or three days in order to measure egg characteristics, to follow embryonic development, and to measure changes in the biochemical composition during development. Images of egg samples were first digitized using a video camera (SPOT INSIGHT) mounted on a stereomicroscope (Leica MZ 75). The eggs were then divided in three sub-samples of 3 to 4 eggs. The fresh weight of each sub-sample was recorded (0.0001 g) and all samples were stored in vials with filtered seawater at –80°C for further analysis.

Image analysis software (Image-Pro Plus, V. 4.1.1.2 ®) was used to determine egg diameters and development stages. An embryonic development key for Greenland halibut (Mejri, 2011), based on developmental stages used for Atlantic cod, *Gadus morhua* (Friðgeirsson, 1978) and American shad, *Alosa sapidissima* (Shardo, 1995), was used to stage the eggs. From fertilization to hatching, 27 embryonic development stages (ED1–27) corresponding to specific events of the embryonic development were used to classify the egg samples (Mejri, 2011). The embryonic development events (Figure 1) were combined into developmental groups for the analysis of egg characteristics and biochemical composition during egg development.

### Egg biochemical composition

The biochemical composition of the eggs was determined for females that spawned in the winter of 2007. Egg dry weight,



**Figure 1.** Egg developmental stages of Greenland halibut. (a) 2-cell stage; (b) gastrula; (c) closure of the blastopore; (d) late neurula, first signs of fins; (e) newly hatched larvae.

protein and lipid contents were determined for the different egg developmental groups incubated at 2, 4, and 6°C. Egg dry weight was determined by drying egg samples in an oven at 65°C for 12 h. For the determination of protein and lipid contents, samples (3 eggs) were homogenized in 1500 µl of NaCl 0.9%. Triplicates of 150 µl aliquots were analysed for total protein content using a spectrometric method (Lowry *et al.*, 1951). BSA (3 mg ml<sup>-1</sup>) was used as a standard and measurements were carried out on a Perkin Elmer Lambda 2 spectrophotometer at 562 nm. Lipids were extracted from duplicate aliquots of 200 µl using the Bligh and Dyer (1959) method. Samples were kept under nitrogen during the extraction procedure. Lipid classes were analysed by thin layer chromatography with flame ionization detection (FID) using silica gel coated Chromarods-SIII and an MK 6 Iatrosan (Parrish, 1987). The separation of total lipids was accomplished with three solvent systems. The first solvent system, hexane/diethyl ether/formic acid (97:3:0.05 v:v:v), was used in a double 30 min development to produce the initial separation of the internal standard (nonadecane) and the esters. A second solvent system, hexane/diethyl ether/formic acid (65:35:0.09 v:v:v) was used (30 min development) for separation of the non-polar lipids (triacylglycerols, free fatty acids and sterols) from the polar lipids. Finally, a third 45 min development in chloroform/methanol/water (30:66:4 v:v:v) solvent system was used for the separation of phospholipids. The surface area of each lipid class on the chromatograms was converted to micrograms of

lipids using calibration curves constructed from solutions of lipid standards (Sigma-Aldrich). The lipid classes identified and quantified were steryl or wax esters, triacylglycerol (TAG), free fatty acids (AGL), diacylglycerol (DAG), sterol (STE), phosphatidil-ethanolamine (PE) and phosphatidil-choline (PC).

Total lipids were also determined with the traditional gravimetric method (Bligh and Dyer, 1959) on larger egg samples as the Iatrosan TLC-FID method for micro quantities has been shown to underestimate total lipids relative to the gravimetric method (Lu *et al.*, 2008). Total lipids were extracted, dried and weighed for newly fertilized 3 g egg samples on a limited number of fish (six individuals).

### Egg buoyancy

Egg specific density was measured through the embryonic development in samples collected in 2007 and 2008. Specific density was determined for egg samples (~10 eggs) of different embryonic development time (days since fertilization). The measurements were done in a salinity gradient column maintained at a temperature of 4–5°C in a cold room (Coombs, 1981). Only eggs incubated at temperatures between 4 and 6°C were used in the density gradient experiments. Egg samples were put on top of the column and their positions recorded following a stabilization period (10–15 minutes) in the gradient and after 1 and 2 h. Only observations recorded after 2 h when egg positions were stable were considered for the analyses. Egg position in the gradient column determined their specific density. The specific density gradient in the column was measured using glass spheres of different known specific gravities calibrated at a temperature of 23°C. A correction factor ( $2.8 \times 10^{-5} \text{ g cm}^{-3}$  for each degree of difference in temperature) was applied to measurements of specific density to account for the difference in calibration (23°C) and experimental (4–5°C) temperature (TECHNE Inc, Density Column, Operator's Manual).

Egg specific density measurements were matched with the density profile of the water column in the Gulf of St Lawrence in order to estimate the vertical position of the eggs at different developmental stages.

### Statistical analysis

Linear regressions on original or log-transformed data were used to study relationships between realized fecundity, egg characteristics, and female length, weight and condition, with condition estimated as Fulton's *K* (somatic weight divided by length cubed). Comparisons of relationships between 2007 and 2008 were done using analysis of covariance (Sokal and Rohlf, 1995). Egg biochemical composition variables were first checked for normal distribution of the data (Kolmogorov-Smirnov test) and homogeneity of variances (Brown-Forsythe's test). The effects of developmental stage and temperature on the biochemical composition were then tested using two-way analysis of variance (ANOVA). When normality of data and homogeneity of variances were not met ANOVA was performed on log-transformed or rank-transformed data (Quinn and Keough, 2002). When significant differences were detected, Tukey-Kramer tests were used for pairwise comparisons (Sokal and Rohlf, 1995).

### Results

Spawning activity in captivity was observed from early January to the end of May, but most of the females spawned between mid-January and mid-April. Of all spawning events observed in

2007 and 2008, respectively, 78% and 69% occurred during that three month period. Following the first ovulation and stripping, none of the females monitored in the recovery tank for a period of two weeks produced a second egg batch, suggesting that the females released all their eggs in a single batch.

No difference in the average size (length) and size range of reproductive females was observed between 2007 and 2008 (Table 1). However, total and somatic weights differed between the two years. The slopes of the relationships between weights (total and somatic) and length (log-transformed data) were not different ( $p > 0.54$ ) but analysis of covariance indicated significantly ( $p < 0.007$ ) higher somatic and total weights in 2008 (Table 1). Weight differences resulted in a significantly (ANOVA,  $p = 0.0008$ ) higher condition factor (Fulton's  $K$ ) in 2008. Despite weight differences between years, no differences in the reproductive characteristics of the females were observed between 2007 and 2008 (Table 1). Realized fecundity increased significantly with both female length and total weight (Figure 2). However, relationships of realized fecundity with both length and weight were not different between years. Realized fecundity–length and realized fecundity–weight relationships did not differ in either of their slopes ( $p > 0.14$ ) or adjusted means (ANCOVA,  $p > 0.24$ ) between 2007 and 2008 (Table 2). The relative realized fecundity, the egg weight index, and the egg fresh weight and diameter were independent of fish size ( $p > 0.059$ ) and comparable between 2007 and 2008 (ANOVA,  $p > 0.31$ ) (Table 1). Finally, female condition (Fulton's  $K$ ) had no effect ( $p > 0.14$ ) on any of these reproductive characteristics.

Hatching time was observed to be strongly dependent on incubation temperature (Figure 3). At 2°C, the time to 50% hatch was 46.3 days, while at 4°C and 6°C, 50% hatching occurred after 30.4 and 23.8 days, respectively. The relationship between the time to 50% hatch ( $H$ , d) and temperature ( $T$ , °C) was best described by  $H = 70.4 T^{-0.606}$  ( $p = 0.0001$ ,  $R^2 = 0.81$ ) for the temperature range tested. Separate analyses for each incubation temperature indicated that female length and condition factor had no significant effect ( $p > 0.21$ ) on the time to 50% hatch. Moreover, the time to attain a specific developmental stage (ED) expressed as a proportion of total development time (i.e. time to hatch) was not influenced by temperature (Figure 4). Thus, the developmental groups combining egg stages had similar relative time duration at all temperatures (Table 3).

Egg dry weight, and protein and lipid contents of early-fertilized eggs (i.e. developmental Group I) were not influenced

by female size and condition. None of the relationships with size or condition were significant ( $p > 0.13$ ). Egg biochemical composition did not show any significant trend during the embryonic development. Moreover, incubation temperature had no significant effect on egg biochemical composition during embryonic development. No significant effect of developmental stage or temperature (two-way ANOVA,  $p = 0.21$ ) was observed on total lipid or protein contents (Figure 5). Egg dry weight did not differ between temperatures (two-way ANOVA,  $p = 0.27$ ), but a significant effect of developmental stage ( $p = 0.003$ ) was observed with higher dry weight in developmental Group II (pairwise comparison,  $p = 0.0004$ ). However, pairwise comparisons did not show any difference between developmental Groups I and IV, or between developmental Groups III and IV ( $p > 0.12$ ). Mean dry weight, total lipid and protein contents ( $\pm$  SD) for combined developmental groups and temperatures were, respectively,  $95.7 \pm 4.9$  ( $n = 74$ ),  $9.0 \pm 3.0$  ( $n = 51$ ), and  $21.6 \pm 3.5$  ( $n = 53$ )  $\mu\text{g mg}^{-1}$  fresh weight. Corresponding values per egg were  $2.33 \pm 0.39$   $\text{mg egg}^{-1}$  for dry weight,  $239.9 \pm 85.9$   $\mu\text{g egg}^{-1}$  for total lipids, and  $564.8 \pm 105.9$   $\mu\text{g egg}^{-1}$  for protein content.

Higher total lipid contents resulted from the gravimetric method using larger egg sample weights. The total lipid content determined for 3 g samples of freshly fertilized eggs ( $n = 6$ ) varied between 12.5 and 13.5  $\mu\text{g mg}^{-1}$  fresh weight with a mean value of  $13.0 \pm 0.05$   $\mu\text{g mg}^{-1}$  fresh weight.

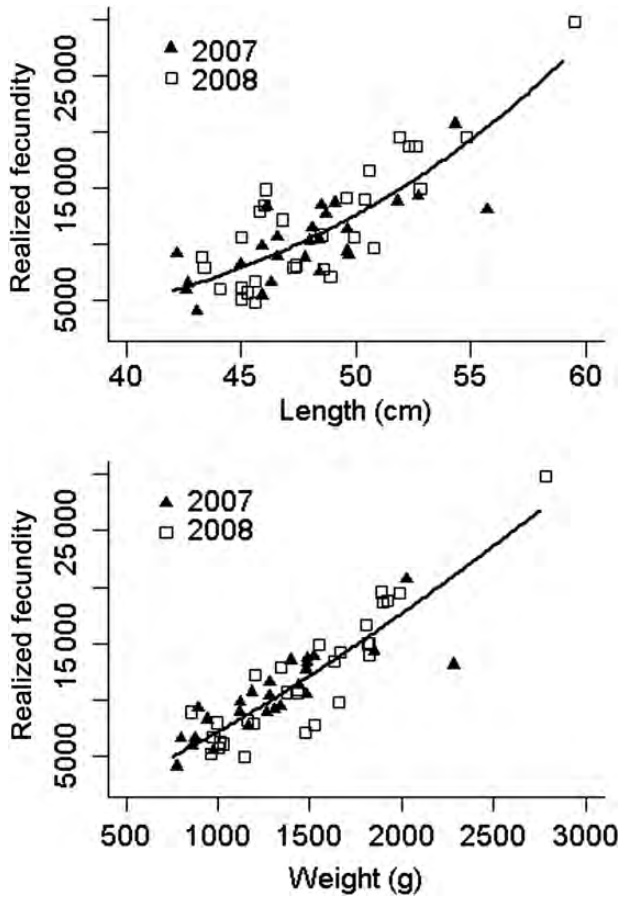
The lipid class composition was not influenced by incubation temperature (ANOVA's on rank-transformed data,  $p = 0.06$ ). Moreover, significant differences between developmental groups were only observed for triacylglycerols and esters where both classes were significantly ( $p < 0.04$ ) higher (in proportion) in developmental Group IV (Table 4). Polar lipids represented 81.5–83.2 % of total lipids, while neutral lipids varied between 16.8 and 18.5%. Phosphatidyl-choline (70.5–74.1%), triacylglycerols (6.5–8.6%), and sterols (6.9–8.9%) were the most abundant lipid classes (Table 4).

Despite the limited changes in the biochemical composition of the eggs, significant changes in egg specific density were observed in relation to development time expressed as the proportion of elapsed time before hatching (Figure 6). Significant variations in specific density were observed in 2007 and 2008 (ANOVA's on rank-transformed data,  $p < 0.0001$ ). Mean egg specific density fluctuated between 1.026 and 1.028  $\text{g cm}^{-3}$  for most of the total embryonic development time (Figure 6). For both 2007 and 2008, significant decreases in egg specific density were observed

**Table 1.** Length, weight, condition factor (Fulton's  $K$ ), fecundity and egg characteristics of Greenland halibut females used in the reproductive experiments conducted in 2007 and 2008.

	2007			2008		
	<i>n</i>	Mean $\pm$ SD	Range	<i>n</i>	Mean $\pm$ SD	Range
Length (cm)	26	47.8 $\pm$ 3.4	42.2 – 55.7	29	48.4 $\pm$ 3.8	43.3 – 59.5
Total weight (g)	26	1 293 $\pm$ 369	774 – 2 280	30	1 479 $\pm$ 425	852 – 2 780
Somatic weight (g)	26	985 $\pm$ 292	606 – 1 932	30	1 139 $\pm$ 302	647 – 2 024
Fulton's $K$	26	0.88 $\pm$ 0.10	0.71 – 1.12	30	0.98 $\pm$ 0.11	0.79 – 1.21
Realized fecundity	26	10 336 $\pm$ 3 535	4 065 – 20 705	30	11 954 $\pm$ 5 648	4 883 – 29 819
Relative fecundity (eggs $\text{g}^{-1}$ )	26	10.5 $\pm$ 2.3	6.6 – 15.1	30	10.2 $\pm$ 2.9	4.9 – 14.7
Egg weight index (% of total weight)	26	23.7 $\pm$ 4.8	15.2 – 33.0	30	22.4 $\pm$ 4.6	13.7 – 2.83
Egg fresh weight (mg)	20	25.7 $\pm$ 2.9	20.7 – 30.3	23	25.4 $\pm$ 2.4	20.4 – 29.1
Egg diameter (mm)	18	3.62 $\pm$ 0.13	3.38 – 3.80	21	3.65 $\pm$ 0.12	3.38 – 3.88

Mean values, SD, range, and number of observations for each year are presented.



**Figure 2.** Realized fecundity in relation to female length and total weight for Greenland halibut in the 2007 and 2008 experiments.

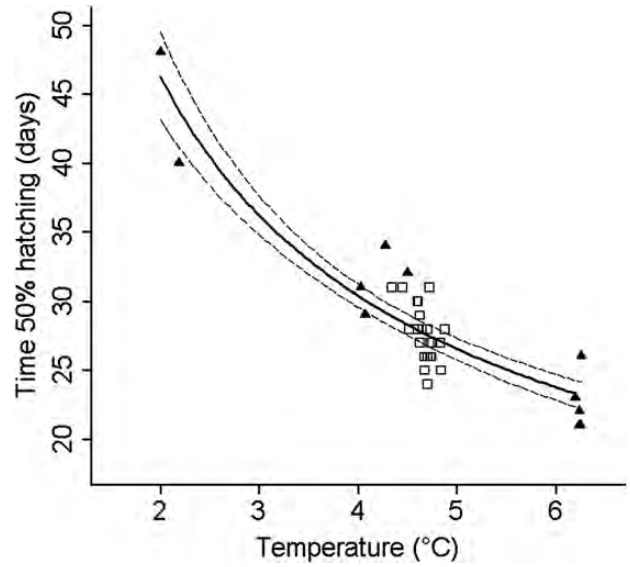
**Table 2.** Regression parameters of realized fecundity ( $F$ )–length ( $\log F = a + b \log L$ ;  $L$  in cm) and realized fecundity–weight ( $\log F = a + b \log W$ ;  $W$  in g) relationships of Greenland halibut in 2007 and 2008 and for combined years.

Year	$a \pm SE$	$b \pm SE$	$R^2$	$p$	$n$
2007	$-2.24 \pm 1.17$	$3.71 \pm 0.70$	0.52	$<0.0001$	26
2008	$-3.91 \pm 1.26$	$4.72 \pm 0.75$	0.58	$<0.0001$	29
2007–2008	$-3.22 \pm 0.86$	$4.30 \pm 0.51$	0.56	$<0.0001$	55
2007	$0.55 \pm 0.40$	$1.11 \pm 0.13$	0.74	$<0.0001$	26
2008	$-0.44 \pm 0.48$	$1.42 \pm 0.15$	0.75	$<0.0001$	30
2007–2008	$0.10 \pm 0.32$	$1.25 \pm 0.10$	0.74	$<0.0001$	56

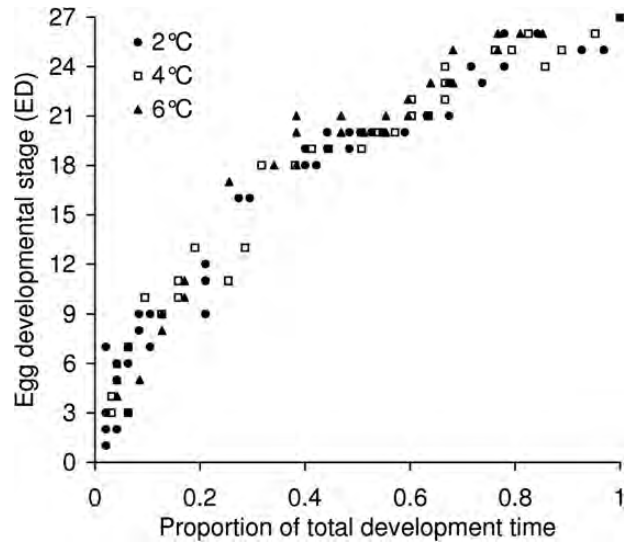
Regression parameters (intercept  $a$  and slope  $b$ ),  $R^2$  = coefficient of determination,  $p$  = probability level of significance,  $n$  = number of fish.

in the last quarter of the development time ( $p = 0.04$ ) for eggs incubated at 4°C and 5°C. Egg specific density decreased during the last period of development to values between 1.025 and 1.027 g cm<sup>-3</sup> (Figure 6).

Hypothetical positions of Greenland halibut eggs in the water column were determined by comparing egg specific density to the density profile of the water column in the Gulf of St Lawrence. The typical density structure of the water column in the eastern part of the Gulf of St Lawrence before the winter season (Figure 7) indicates a density stratification of the water with a rapid increase from a minimum in seawater specific density of 1.0245 g cm<sup>-3</sup> at the surface to a value of 1.0267 g cm<sup>-3</sup>



**Figure 3.** Time in days at 50% hatching for Greenland halibut eggs incubated at temperatures between 2°C and 6°C in 2007 (triangles) and 2008 (squares). The relationship is described as a power function (solid line) with the 95% confidence intervals (dotted lines). Each point represents hatching time for the egg batch of individual females.

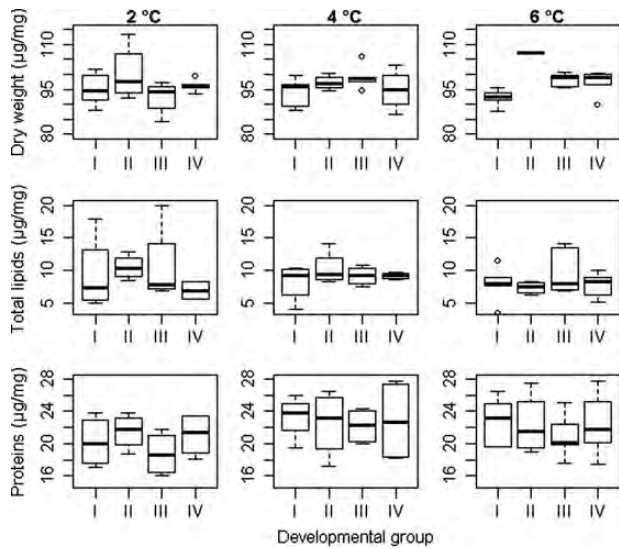


**Figure 4.** Embryonic development of Greenland halibut at different temperatures expressed as the proportion of total development time needed to reach egg developmental stage (ED1 to ED27) at 2, 4, and 6°C.

at ~150 m. The specific density below 150 m continues to increase with depth and reach a maximum of 1.0275 g cm<sup>-3</sup> at 400 m. The positions of the eggs based on their specific density at different development time (grouped data for 2007 and 2008) along the density gradient of the water column indicate that the eggs would be neutrally buoyant at depths below 300 m for 80% (i.e. ~21 days at 5°C) of the development time before hatching. However, in the last 20% of the development time, the eggs would be neutrally buoyant at depths of between 100 and 230 m

**Table 3.** Classification of embryonic developmental (ED) stages of Greenland halibut into developmental groups and relative time to hatching (i.e. proportion of elapsed time before hatching) for each group.

Developmental group	Egg stage	Relative time to hatching
I	ED1–13 : Fertilization to the beginning of gastrulation	0.00–0.25
II	ED14–19: Gastrulation to the stage before the closure of the blastopore	0.25–0.50
III	ED20–22 : Closed blastopore	0.50–0.75
IV	ED23–27: First signs of fins to hatching	0.75–1.00



**Figure 5** Box-plots of Greenland halibut egg dry weight, total lipids, and protein contents ( $\mu\text{g mg}^{-1}$  fresh weight) for the different developmental groups (defined in Ttable 1) incubated at 2°C, 4°C, and 6°C. The boxes represent the 25th and the 75th percentiles with the median inside. Whiskers below and above boxes indicate the 10th and the 90th percentiles.

(Figure 7), the lowest egg densities being observed for the latest developmental stages (Figure 7).

**Discussion**

Most of the spawning activity of Greenland halibut in captivity took place within a three month period between January and March. These results are consistent with observations of prespawning concentrations of Greenland halibut in the Gulf of St Lawrence in January–February (Bowering, 1982) as well as spawning time (March–April) estimated from larval development and size and from newly settled post-larvae sampled between April and August in the Gulf of St Lawrence (Ouellet *et al.*, 2011). Although not precisely determined, similar spawning time and duration have also been observed or estimated in other Greenland halibut stocks (Albert *et al.*, 2001; Morgan *et al.*, 2003; Kennedy *et al.*, 2011). However, year-round spawning has also been hypothesized in some of these stocks (Fedorov, 1971; Junquera and Zamorro, 1994; Morgan *et al.*, 2003). The imprecision in determination of spawning time may come from the fact

that spawning fish are rarely caught by trawlers or longliners (Kennedy *et al.*, 2009). The very short spawning duration may largely explain the difficulty in catching Greenland halibut with hydrated eggs. The monitoring of reproductive females of Greenland halibut in the present study indicates that this species can be classified as a group-synchronous, determinate, and total spawner (Murua and Saborido-Rey, 2003). The results confirm the hypothesis that hydration of all yolked oocytes from the leading cohort occurs at the same time with all ovulated eggs released in one reproductive event. Following observations of females that were closely monitored for two weeks following the first spawning event, it cannot be excluded that a second egg batch could be produced over a longer time interval. However, the comparison of realized fecundity in the present study with measures of potential fecundity of Greenland halibut in the Gulf of St Lawrence (Bowering, 1980) supports the hypothesis of a single egg batch for this species.

The relationship observed between realized fecundity and length is similar to the relationship between potential fecundity and length measured for Greenland halibut caught in January 1978 in the Gulf of St Lawrence (Bowering, 1980). Realized fecundity for a 55 cm fish in the present study matches the potential fecundity of 18 574 eggs observed by Bowering (1980). Higher potential fecundities were obtained in 1999 and 2000 (23 635 and 28 885 eggs, respectively) for similar female size (B. Morin and B. Bernier, Fisheries and Oceans, Maurice Lamontagne Institute, Mont-Joli, QC, Canada, unpublished data). These differences may result from annual variations in potential fecundity, or potential fecundity may have been overestimated as measurements were done in August, several months before spawning in winter. Down-regulation of fecundity in Greenland halibut has been observed with decreases in estimates of potential fecundity between 17 and 45% for the period between August and March of the following year (Kennedy *et al.*, 2009).

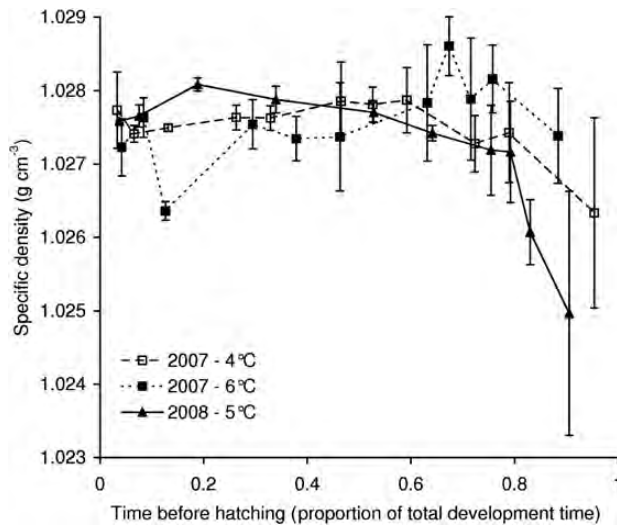
Realized fecundity could also have been underestimated due to some atresia caused by the capture and transport of fish and by the two to four weeks period taken by the fish to resume feeding following capture. However, based on the maturation cycle described by Gundersen *et al.* (2001a), the leading cohort of oocytes of Greenland halibut captured in October in the present study should have reached a late vitellogenic stage when atresia was observed to be infrequent (Tuene *et al.* 2002). Moreover, given the absence of an effect of Fulton’s condition factor and liver index on the potential fecundity of Greenland halibut (Kennedy *et al.* 2009), a two to four weeks fast should not have a major impact on fecundity unless these proxies are not correlated in any way with energy reserves. Finally, the realized fecundity measured in the present study is well within the range of observed potential fecundities of Greenland halibut of similar sizes found in the Flemish Cap, Greenland, Iceland, Barents Sea, and the Faroe Islands (Junquera *et al.*, 1999; Gundersen *et al.*, 2000; Gundersen, 2002; Gundersen *et al.*, 2002a; Gundersen *et al.*, 2002b; Gundersen *et al.*, 2009).

Egg diameter and fresh weight were constant for the size range of female Greenland halibut used in the present study. Similar egg diameters were observed for artificially spawned eggs of a 70 cm female (3.2–4.2 mm) from the Barents Sea (Stene *et al.*, 1999). However, slightly higher egg diameters have been reported from field surveys in the Barents Sea, Greenland, and Iceland (3.7–4.7 mm), which might indicate a possible effect of female size on egg diameter. Indeed, in these areas the mean size of

**Table 4.** Lipid class composition as % of total lipids (and SD) of Greenland halibut eggs for the different developmental groups defined in Table 1.

Lipid classes	I	II	III	IV
Polar lipids	82.1 ± 4.1	83.2 ± 3.2	82.4 ± 3.9	81.5 ± 1.9
Phosphatidyl-choline	70.5 ± 7.9	74.1 ± 6.3	73.3 ± 3.7	72.2 ± 4.0
Phosphatidyl-ethanolamine	11.6 ± 4.7	9.1 ± 4.7	9.2 ± 2.6	9.3 ± 3.4
Neutral lipids	17.9 ± 4.1	16.8 ± 3.2	17.6 ± 3.9	18.5 ± 1.9
Triacylglycerols	7.1 ± 1.5 <sup>a</sup>	6.5 ± 1.5 <sup>a</sup>	7.4 ± 2.2 <sup>a</sup>	8.6 ± 1.5 <sup>b</sup>
Sterol	8.9 ± 3.5	8.0 ± 2.1	7.5 ± 1.7	6.9 ± 0.8
Steryl or wax esters	1.3 ± 0.7 <sup>a</sup>	1.4 ± 0.5 <sup>a</sup>	1.6 ± 0.7 <sup>a</sup>	2.0 ± 0.6 <sup>b</sup>
Free fatty acids	0.6 ± 0.4	0.8 ± 0.4	1.0 ± 0.5	1.0 ± 0.5
Diacylglycerols	0.01 ± 0.03	0.04 ± 0.09	0.01 ± 0.04	0.02 ± 0.06

Letters indicate significant differences in lipid classes between developmental groups.

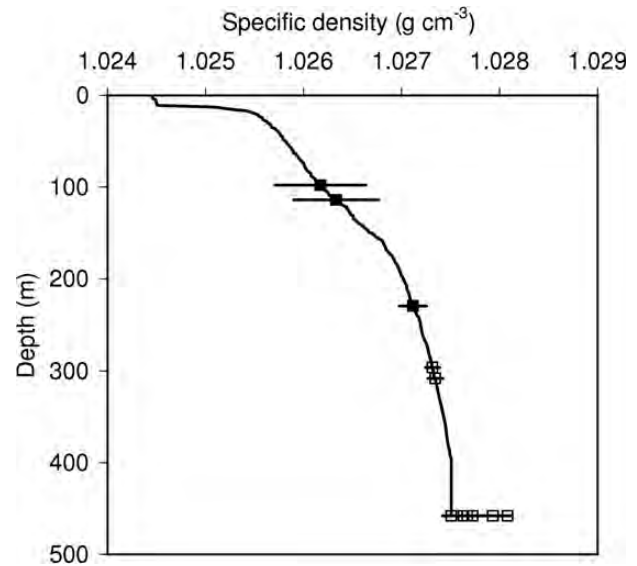


**Figure 6.** Egg specific density variations during the embryonic development of Greenland halibut for eggs incubated at temperatures between 4°C and 6°C. Development time is expressed as elapsed time in proportion to total time from fertilization to 50% hatching.

mature females is higher, with few mature fish having a size below 60 cm (Morgan *et al.*, 2003). On the other hand, the fertilization process and environmental conditions in captivity could influence egg diameter (Stene *et al.*, 1999). For example, smaller size of artificially spawned eggs was observed for Atlantic halibut, *Hippoglossus hippoglossus* (Lønning *et al.*, 1982).

The results showed that female condition did not influence egg diameter and fresh weight, relative fecundity or egg weight index. However, the observed limited range in female condition in the present study might not be large enough to detect a possible effect of condition on fecundity and egg characteristics. Moreover, in the absence of studies on the relationship between the energy content and the condition factor for Greenland halibut, it cannot be certain that the condition factor for this species is a good proxy of the energetic condition (Kennedy *et al.*, 2009).

The present study represents the first estimation of the egg development time in relation to temperature for Greenland halibut. The only previous information available for this species was obtained from the incubation of the eggs of one female at ~2°C (Stene *et al.*, 1999). According to their study, the eggs (only eight eggs) hatched 53 days after fertilization, which is



**Figure 7.** Typical density gradient profile of the water column in the Gulf of St. Lawrence, and egg positions at depths of neutral buoyancy. Depth distribution based on egg specific density (mean and SD) is presented for egg samples representing developmental stages occurring during the first 80% of the total development time (□) (open squares) and the last 20% of the total development time (■) (closed squares).

slightly greater than the time to 50% hatch observed in the present study (46 days). As expected, egg development was slower at lower temperature, with time for 50% hatching being almost twice as long at 2°C than at 6°C. Egg development time is also longer than in Atlantic halibut, a species with similar egg characteristics. The time to 50% hatch in Greenland halibut is 44 and 70% longer than in Atlantic halibut at 3°C and 6°C, respectively (Pittman *et al.*, 1990).

According to our results, Greenland halibut can be identified as a species with long incubation time, large egg size, and lipid-poor eggs. Based on size frequency distribution in egg diameter of marine fish species, the egg diameter of Greenland halibut is one of the largest observed (Kamler, 2005). Generally, high total lipid content of eggs is associated with the presence of oil globules, basically made up of neutral lipids (Henderson and Tocher, 1987). This type of egg usually belongs to species with long incubation periods. The absence of oil globules, the relatively lower total lipid content (9.4–14.1%), and the high contribution of

phospholipids to total lipids ( $\sim 82\%$  of total lipids), classifies Greenland halibut as a lipid-poor egg species (Kamler, 1992; Wiegand, 1996). The biochemical composition of Greenland halibut eggs observed in the present study is similar to that of Atlantic halibut. Mean dry weight and lipid content per egg (1.8–2.2 mg DW per egg and 220–320  $\mu\text{g}$  lipids per egg), and the proportions of PL, TAG, and sterols (76%, 10%, and 8%, respectively) in Atlantic halibut (Falk-Petersen *et al.*, 1989; Evans *et al.*, 1996) are in the same range as those observed for Greenland halibut in the present study. Moreover, as observed for Atlantic halibut, egg dry weight, lipid and protein contents of Greenland halibut were found to vary little during egg development (Finn *et al.*, 1991). The low metabolic requirements of the embryo may explain the relative stability in biochemical composition throughout the embryonic development. From the total oxygen consumption over the development period, it was estimated that 0.4–0.5 J would be lost from aerobic catabolism during development in Atlantic halibut, while total egg energy content was estimated to vary between 32 and 41 J egg<sup>-1</sup> (Finn *et al.*, 1991). The absence of changes in lipids and proteins could also indicate that the energy for development may come from other sources like free amino acids (FAA) which decrease during egg development in turbot (*Scophthalmus maximus*), gilthead sea bream (*Sparus aurata*), and Atlantic halibut (Rønnestad *et al.*, 1992; Rønnestad *et al.*, 1993; Rønnestad *et al.*, 1994; Zhu *et al.*, 2003). The hatching at a very early stage of embryonic development in winter or early spring may also reflect a strategy for a long development time of the larvae using endogenous reserves until the initiation of the spring bloom and secondary production.

While the absence of significant changes in the biochemical composition during egg development could explain the stability in egg specific density during most of the embryonic development, other factors such as FAA, ion concentrations and water content could be responsible for the important change in the egg buoyancy observed just before hatching. It has been demonstrated that FAA and ion concentrations are important for the regulation of fish egg osmolarity and buoyancy (Craig and Harvey, 1987; Fyhn, 1993; Thorsen *et al.*, 1993; Thorsen *et al.*, 1996; Rønnestad *et al.*, 1998). In Baltic cod, it was reported that near hatching, environmental factors had larger effects than egg biochemical composition on egg buoyancy (Nissling and Vallin, 1996). In the present experiment, mean egg specific density is similar to laboratory observations made by Stene *et al.* (1999) on Greenland halibut. Seawater densities corresponding to neutral buoyancy of the eggs are generally found in depths where eggs can be considered mesopelagic. In the Gulf of St Lawrence, the typical density gradient profile of the water column suggests that eggs would be neutrally buoyant at depths greater than 300 m during most of their development. In the Barents Sea, seawater density corresponding to neutral buoyancy was found at depths of around 650 m (Stene *et al.*, 1999). Moreover, Greenland halibut eggs sampled in field surveys, although limited in number, also indicated a mesopelagic distribution of the eggs (Stene *et al.*, 1999).

The decrease in egg specific density just before hatching also suggests that early larval stages will be found higher in the water column. Mean egg specific density during late developmental stages indicates neutral buoyancy at depths between 100 and 225 m in the Gulf of St Lawrence. In many studies, newly hatched larvae have been reported as being mesopelagic, with eggs hatching in deep waters and rising in shallower waters as they develop (Smidt, 1969; Bulatov, 1983; Sohn *et al.*, 2010).

However, the present study suggests that eggs will rise in the water column during late egg developmental stages (i.e. the last 20% of development time), and that hatching will occur in shallower depths.

Our results provide valuable new knowledge on the reproduction and early life stages of Greenland halibut in the Gulf of St Lawrence, where limited information is available. The spawning area for that stock is not well defined, but adult fish distribution from winter surveys conducted at the end of the 1970s indicate large concentrations of fish in the eastern part of the Gulf of St Lawrence at the beginning of the expected spawning period for that stock (Bowering, 1982). Based on the bottom temperature in that area (5°C) at depths corresponding to neutral buoyancy, egg incubation time would be 27 days. During that period, limited displacement of the eggs would be observed due to the low residual near-bottom westward current (Ouellet *et al.*, 2011). Hatching would occur in shallower depths at temperatures of between 0 and 3°C. No information on larval growth and distribution between hatching and the complete resorption of the yolk sac is available. However, preliminary data suggest that hatching occurs at a size of  $\sim 10$  mm, and that complete resorption of the yolk sac at 4–5°C is observed after 50 to 60 days, when larval size reaches  $\sim 15.5$  mm (YL, unpublished data). Ichthyoplankton surveys in the Gulf of St Lawrence reported catches of Greenland halibut larvae in the 15–20 mm size range at depths of between 0 and 50 m in the April–May period (Ouellet *et al.*, 2011), suggesting that major spawning activity would occur before March, and that a net westward displacement of the larvae in the upper water column would be observed. However, further work on larval development and behaviour at different temperatures is needed to fully understand drift pathways of the larvae to the nursery areas located in the west part of the Estuary and Gulf of St Lawrence.

Population connectivity (i.e. exchange of individuals between areas) has important consequences for recruitment success and population productivity. In marine species, the understanding of processes driving early life stage dispersal patterns from spawning grounds to nursery areas is necessary before patterns of population connectivity can be better described (Cowen and Sponaugle, 2009). In Greenland halibut, the modelling of dispersion and drift pathways of eggs and larvae from spawning grounds to nursery areas relies on the accurate determination of egg and larval development time and distribution in relation to specific environmental conditions (Ådlandsvik *et al.*, 2004; Sohn *et al.*, 2010). The results of the present laboratory experiments provide highly relevant information for the development and improvement of these models, which will lead to a better understanding of Greenland halibut population dynamics.

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