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Evaluating the use of the autodiametric method for estimating fecundity of *Reinhardtius hippoglossoides*, a species with an unusual oocyte development strategy

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The autodiametric method is a highly streamlined method for estimating fecundity of fish with determinate oocyte development pattern. Greenland halibut presents a peculiar reproductive strategy with two simultaneously cohorts one of large vitellogenic oocytes (for the current year) and another one of small vitellogenic oocytes (for the subsequent year). Results of this study showed that autodiametric method can be applied to estimate fecundity in Greenland halibut. Additionally, spatial differences in the autodiametric calibration curve were observed in the Northwest Atlantic, but did not translate into differences in fecundity at length. This is the first time that spatial differences between ACCs of the same species have been reported, what could be the result of (i) the unusual oocyte development pattern, or (ii) spatial differences in oocyte biochemistry. More research on the relative dynamics of oocyte cohorts simultaneously present in Greenland halibut ovaries and the factors (endo- or exogenous) influencing oocyte packing density could provide a better understanding of observed geographical differences.

Keywords: autodiametric method, fecundity, Greenland halibut, Northwest Atlantic, reproductive potential.

Introduction

The estimation of fish fecundity is an important aspect for studying fisheries ecology, allowing the exploration of spawning energetics and the reproductive dynamics of fish populations, and to estimate annual reproductive output and links to subsequent recruitment (Ganias *et al.*, 2014). However, the time-intensive nature of traditional fecundity estimation techniques have proved challenging for long-term monitoring programs (Tomkiewicz *et al.*, 2003). These traditional techniques generally involved counting a subsample of the eggs in the ovary and then scaling that number up to the total potential fecundity (PF) based on the proportion of the total weight or volume of the gonad that was sampled. The bottleneck of these methods was primarily related to the techniques used to liberate oocytes from the ovarian tissue (e.g. physically teasing oocytes from connective tissue, treatment with chemicals to dissolve connective tissue, and/or rinsing tissue through a series of graduated sieves to collect the oocytes) and the typically manual counting of oocytes.

The autodiametric method (Thorsen and Kjesbu, 2001) is a highly streamlined method for estimating fish fecundity. The

method relies on a relationship between mean vitellogenic oocyte diameter and oocyte packing density (OPD). Mean oocyte diameter of an ovarian subsample is quickly obtained using an automated image analysis system and then converted to OPD, which is then scaled up to give an estimate of fecundity based on the weight of the ovaries (Ganias *et al.*, 2014). This method has been widely utilized for estimating fecundity on a broad range of species with determinate fecundity (Murua *et al.*, 2003; Boulcott and Wright, 2008; Kennedy *et al.*, 2008; Lambert, 2008; Alonso-Fernández *et al.*, 2009; Witthames *et al.*, 2009). The suitability of the autodiametric method for use on species with indeterminate fecundity has been questioned (Witthames *et al.*, 2009); these species have a single continuous oocyte size distribution, which overlaps with the previtellogenic distribution leading to a high variance in the estimation of oocyte density hampering accurate prediction of fecundity with this method (Witthames *et al.*, 2009). However, a technical expansion of the autodiametric method (incorporating aspects of stereology) was used to examine oocyte recruitment in at least one species with indeterminate fecundity (Korta *et al.*, 2010). Clearly the suitability of the autodiametric method for estimating fecundity depends on oocyte development patterns and should be examined on a per species basis (Witthames *et al.*, 2009).

Greenland halibut *Reinhardtius hippoglossoides* is a deepwater flatfish found in the Northern hemisphere in both the Atlantic and Pacific oceans. In the Northwest Atlantic around Newfoundland, Greenland halibut are managed as three stocks, with those in the Gulf of St Lawrence (GSL) considered separate from the much larger stock off the east coast (Arthur and Albert, 1993) and the northern stock between Canada and Greenland (Arthur and Albert, 1993). Unlike many other flatfish, Greenland halibut are highly active predators, with reduced substrate association and often undertaking substantial migrations (de Groot, 1970; Bowering, 1984; Bowering and Lilly, 1992; Boje, 2002; Vollen and Albert, 2008; Dennard *et al.*, 2009). Noteworthy reproductive attributes include highly variable and difficult to interpret maturation schedules (Morgan and Bowering, 1997) as well as suggestions of non-annual spawning (Fedorov, 1971; Junquera *et al.*, 2003) . Greenland halibut has generally been considered to have determinate fecundity with group synchronous oocyte development (Gundersen *et al.*, 1999; Junquera *et al.*, 1999; Tuene *et al.*, 2002). However, the simultaneous presence of two cohorts of vitellogenic oocytes, one large and one small, has led to much confusion, as the fate of the smaller vitellogenic oocytes was uncertain (Fedorov, 1968; Saborido-Rey and Junquera, 1998; Rideout *et al.*, 1999). More recently it has been suggested that Greenland halibut utilize a very unusual oocyte development pattern, whereby an individual cohort of developing oocytes requires >1 year to complete vitellogenesis, but the potential for annual spawning is maintained by the fact that developing ovaries contain two simultaneously developing cohorts of vitellogenic oocytes. The larger oocytes are developing to spawn in the current year, while the cohort of smaller vitellogenic oocytes are developing for the subsequent reproductive season (Gundersen *et al.*, 2000; Kennedy *et al.*, 2011; Rideout *et al.*, 2012). In captivity, Greenland halibut females produce a single batch during the spawning season, i.e. it is a total spawner (Dominguez-Petit *et al.*, 2013)

Previous fecundity studies have presented data for Greenland halibut from a broad range of geographical areas, including the Barents Sea (Gundersen *et al.*, 1999, 2000; Núñez *et al.*, 2015), East Greenland (Gundersen *et al.*, 2001; Kennedy *et al.*, 2009), West Greenland (Gundersen *et al.*, 2000), Iceland (Gundersen *et al.*, 2009), Bering Sea (D'yakov, 1982), Flemish Cap (FC) (Junquera *et al.*, 1999), the GSL (Bowering, 1980), and the Labrador-eastern Newfoundland area (Lear, 1970; Bowering, 1980; Serebryakov *et al.*, 1992). Some studies have attempted to count only those oocytes of the larger vitellogenic size class by employing size selection criteria (e.g. Junquera *et al.*, 1999; Gundersen *et al.*, 2000; Núñez *et al.*, 2015), while others have counted all vitellogenic oocytes (e.g. Lear, 1970) or provided insufficient detail to fully evaluate oocyte size selection criteria (e.g. Bowering, 1980; Serebryakov *et al.*, 1992). Such differences in methodology raise questions about the comparability of historically collected fecundity data. The unusual oocyte development pattern employed by Greenland halibut also raises questions about the suitability of the autodiametric method for estimating fecundity for this species. Previous studies (e.g. Núñez *et al.*, 2015) have used automated particle counting to improve over previous manual counting procedures but the relationship between oocyte size and OPD has not been explored. This relationship is the foundation of the autodiametric method and could make future fecundity work on this species much more efficient by requiring only gonad weight and average oocyte size in order to estimate fecundity.

The first objective of this study was to determine if the autodiametric method could be reliably used for estimating fecundity of Greenland halibut, despite the unusual oocyte development pattern (i.e. presence of a confounding secondary group of vitellogenic oocytes). Second, autodiametric calibration curves (ACCs) were developed and compared for fish collected from four general areas in the Northwest Atlantic: FC (NAFO Div. 3 M), Grand Bank (GB) (NAFO Divs. 3LNO), Southern Newfoundland (SNF) (NAFO Subdiv. 3Ps) and the GSL (NAFO Div. 4 S). Finally, these ACC were used to estimate PF of Greenland halibut from the various areas and test for regional differences.

Material and methods

This study brings together work done by three different laboratories to examine fecundity of Greenland halibut from four different areas in the Northwest Atlantic. Data from the GSL were collected by the Institute Maurice Lamontagne (IML, DFO, Canada). Data from SNF (NAFO Subdiv. 3Ps) were collected by the Northwest Atlantic Fisheries Centre (NAFC, DFO, Canada). Data from the FC (Div. 3 M) were collected by the Oceanographic Centre of Vigo (IEO, Spain) and data from the eastern edge of the GB (Divs. 3LNO) were collected by both NAFC and IEO.

Ovaries with advanced vitellogenic oocytes were collected from a total of 156 female Greenland halibut. Fork length and gonad weight were recorded for all fish. Fish from the GSL (n = 55) had fork lengths of 38–74 cm. Of these, 41 specimens were caught in October 2007 and 14 in August 2008 in the St Lawrence estuary and the area of the Laurentian Channel and Anticosti Island (Figure 1). Fish from SNF d (n = 21) were collected in July 2010 from commercial gillnets and had fork lengths of 60–88 cm. Fish were collected from the eastern slope of the GB by both NAFC (n = 35) and IEO (n = 21). Analyses indicated no difference in the oocyte size–density relationship between samples collected and processed by the two labs and hence these samples were combined as "GB". The NAFC samples were collected in July–August 2010 from commercial gillnet vessels and had fork lengths of 56–91 cm, while the IEO samples were collected between February 2001 and August 2009 during both scientific and commercial

surveys and had fork lengths of 51–95 cm. Fish from the FC (3 M) were collected in June–August 2004–2008 from scientific surveys (fork lengths of 60–98 cm) and in April 2007 and 2008 from commercial surveys (fork lengths of 58–89 cm).

Ovaries to be used for fecundity analyses were either fixed onboard the vessel (IML, IEO) or were left in the gutted fish carcass (0–2 days) on ice until landing (NAFC). For ovaries fixed at sea, subsamples were taken by teasing out a small piece of tissue using pointed forceps, while for the non-fixed ovaries; subsamples of fresh tissue were collected with a positive displacement pipette and subsequently fixed. Table 1 summarizes sampling procedures in each lab. The fixative used by all three laboratories was 4% phosphate buffered formaldehyde. Care was taken to avoid collecting ovarian wall tissue as part of the subsamples. The gonad subsample weight varied between 0.03 and 3.4 g depending on the method used for subsampling and the developmental stage of the ovaries: more tissue was needed from ovaries with larger oocytes in order to get a sufficient number of oocytes to be representative of the whole gonad (Gundersen and Emblem, 2002). Oocyte counts and sizes were obtained by examining the ovarian subsample whole-mounts via an image analysis system and the freeware ImageJ or software Leica QWin, depending on the lab.

A preliminary examination of oocytes from six fixed locations within Greenland halibut ovaries (distal, central and proximal position to gonoduct, in both left and right lobes) revealed no significant difference in OPD throughout the ovaries and so any potential influence of sample collection site within the gonads was considered negligible. Also, for those laboratories that collected subsamples from whole ovaries fixed at sea, the use of a fresh-fixed weight relationship to estimate "fresh" weights was investigated. This conversion did not significantly influence results ($r^2 = 0.99$, $p < 0.01$) and hence it was decided to proceed using the weights as collected by each laboratory (i.e. fixed weights for IML and IEO; fresh weights for NAFC).

The unusual oocyte development strategy employed by Greenland halibut poses challenges for estimating fecundity. Ovaries in advanced stages of development contain two cohorts of vitellogenic oocytes. The cohort of larger vitellogenic oocytes (G1) is considered to represent the PF for the current spawning year. The smaller vitellogenic oocytes (G2) are thought to be developing for the subsequent year. Hence distinguishing between the two cohorts of vitellogenic oocytes is a necessity for accurately estimating fecundity. During the early stages of vitellogenesis, only a single mode of vitellogenic oocytes is observed (G2). Later in development, the largest of these vitellogenic oocytes begin to develop at an accelerated pace, resulting in two modes of vitellogenic oocytes with a hiatus in the size distribution between the two (Rideout *et al.*, 1999). Because the G1 oocytes develop at a greater rate than the G2 cohort the size of the hiatus between the two cohorts increases (Kennedy *et al.*, 2011). In the samples that we examined, the hiatus that distinguished the two cohorts of vitellogenic oocytes first formed when G1 reached ~1000 μm. The largest G2 oocytes that we observed (i.e. those on the lower side of the size hiatus) were ~700 μm. These findings led to the generalization that G2 oocytes are typically <700 μm. The two cohorts are easily distinguishable when whole-mount preparations are viewed with transmitted light (Figure 2). Oocytes of the G2 cohort have limited quantities of yolk in the cytoplasm and are translucent to transmitted light, giving them a brownish appearance. The larger G2 oocytes, however, are packed with yolk, making them opaque to transmitted light and appearing as black spheres (Rideout *et al.*, 1999; Gundersen and Emblem, 2002; Simonsen and Gundersen, 2005) (Figure 2). To evaluate ACCs for Greenland halibut only the most advanced vitellogenic oocytes in the ovaries were counted. For ovaries in the later stages of development these would have been G1 oocytes (i.e. >1000 μm). In those samples where oocytes between 700 and 1000 μm represented the most advanced oocytes in the ovaries, G1 was not clearly separated from G2, and all oocytes >700 μm were counted and measured to estimate ACC. No G2

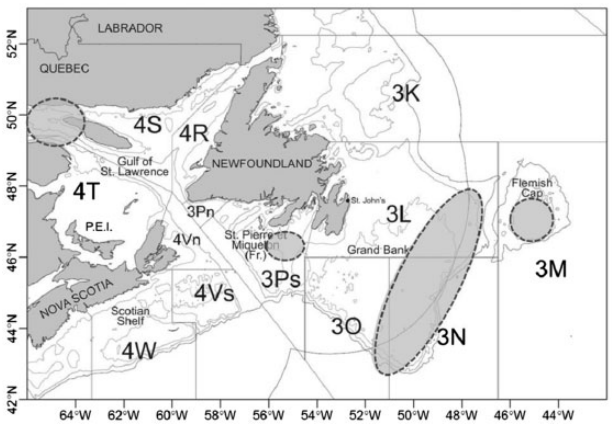

Figure 1. Map of the sampling areas (DFO. http://www.dfo-mpo.gc.ca/international/media/images/nafo_map-eng.jpg).

Table 1. Summary of sampling procedure in each laboratory.

Laboratory	Area	Period	Years	Survey type	N	Size range (cm)	Sampling method
IML	GSL	Oct	2007	Scientific	41	33–61	A
	GSL	Aug	2008	Scientific	14	38–73	A
NAFC	SNF	Jul	2010	Commercial	21	60–88	B
	GB	Jul-Aug	2010	Commercial	35	56–91	B
IEO	GB	May-Aug	2001-2009	Scientific	10	51–95	A
	GB	Feb-Aug	2001-2006	Commercial	11	68–84	A
	FC	Jun-Aug	2004-2008	Scientific	14	60–98	A
	FC	Apr	2007-2008	Commercial	10	58–89	A

Sampling method is codified as A: when whole gonad was fixed immediately onboard and a piece of fixed tissue (0.15–3.4 g) was removed at laboratory for fecundity analysis, and B: when gutted fish with gonad inside were preserved on ice until arrive to the laboratory, small biopsy (0.03–0.18 g) of fresh gonad was taken with a pipette and then fixed for fecundity analysis. IML, Institute Maurice Lamontagne (DFO, Canada); NAFC, Northwest Atlantic Fisheries Centre (DFO. Canada); IEO, Spanish Institute of Oceanography (Spain); GSL, Gulf St Lawrence; SNF, South Newfoundland; GB, Grand Banks; FC, Flemish Cap.

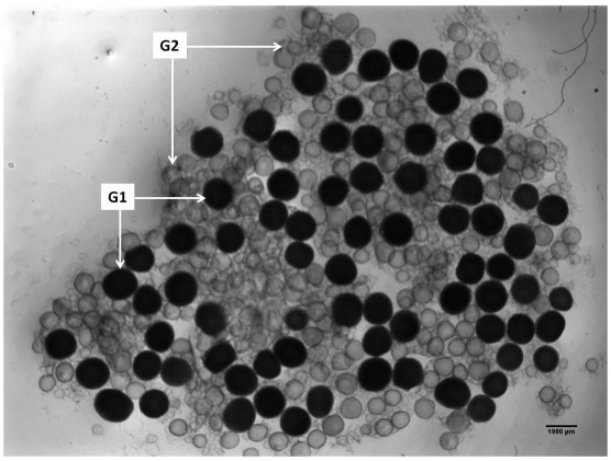

Figure 2. Whole mount gonad sample. G1, cohort to be released in the current spawning season (>1000 μm) and G2, cohort to be released in the next spawning season (<1000 μm). Oocytes between 700 and 1000 μm are opaque and were also included in the ACC when G1 oocytes were not evidently present. Reference bar correspond to 1 mm.

oocytes were counted if G1 oocytes were present (i.e. only the most advance oocytes were counted).

ACCs were estimated according to the Thorsen and Kjesbu (2001) protocol based on Fry (1949) for each study area and lab:

$$\mathrm{OPD} = \mathrm{a} \cdot \mathrm{MD}^{\mathrm{b}},$$

where OPD refers to oocyte packing density in the gonad (number of oocytes/g of gonad) and MD to mean diameter of the most advanced mode of vitellogenic oocytes (μm).

Natural logarithmic transformation was applied to the data to achieve linearity; despite transformation data were not normally distributed, so the Fligner-Killeen non-parametric test was used to test homocedasticity of data ($p > 0.01$). GLMs were used to test homogeneity of slopes by introducing the interaction term (LnMD*Area) in the model. After testing all the assumptions, Analysis of Covariance was used to compare curves among the four study areas, with the dependent variable being the oocyte packing density (LnOPD), the continuous variable the oocyte mean diameter (LnMD) and the covariate the study area. Interactions were tested and no differences in the slopes of the regression lines were observed, so the analysis was exclusively focused on the main effects (Ln*OD – LnMD + Area*). Akaike Information Criterion (AIC) was used to determine relative quality of statistical models. Goodness of fit of the model was estimated as:

$$\mathrm{r}^2 = 1 - (\text{residual deviance}/\text{null deviance}).$$

The PF for each fish was estimated based on oocyte density (oocytes·g^{-1}) and the total weight of the ovaries. A PF–length relationship was estimated in each area and regional differences were tested by fitting data to the GLM *PF*~*L* + *Area*. Goodness of fit of the model was calculated as explained above. Tukey's range test was used for *post hoc* pair-wise comparisons. In all analyses, differences were considered statistically significant when the *p*-value was < 0.01.

Given the unusual oocyte development pattern used by Greenland halibut, the ACCs developed here were examined in relation to those estimated for other species with more standard determinate oocyte development patterns (e.g. *Sebastes spp.*, European plaice, Atlantic cod and Atlantic herring—data from Witthames *et al.*, 2009).

Results

The observed range of MD was variable among areas, ranging from 803 to 1388 μm for the FC, 710–2153 μm for the GB, 981–2143 μm in the Gulf St Lawrence, and 820–1483 μm SNF d. Observed OPD ranges were 469–1840 oocytes·g^{-1}, 129–2673 oocytes·g^{-1}, 113–900 oocytes·g^{-1} and 473–1616 oocytes·g^{-1} for FC, GB, GSL, and SNF, respectively.

In all areas, a negative power function provided a significant fit to the relationship between OPD and MD (Figure 3a). Parameters of ACCs from each area are shown in Table 2. Not surprisingly, the best fits correspond to those areas with the highest sample size and largest range of MD, i.e. GB and GSL.

Regional differences in ACCs were statistically significant. This was true regardless of whether comparisons were based on the full range of observed oocyte diameters ($r^2 = 0.96$, AIC: −147.8, df = 151. Figure 3a) or only the overlapping range ($r^2 = 0.88$, AIC: −115.3, df = 93) (Figure 3b). *Post hoc* analysis showed no significant difference in ACCs between the FC and GB ($p > 0.05$). However, these two areas were statistically different from the GSL ($p < 0.01$) and SNF ($p < 0.01$) and those two areas differed from each other as well ($p < 0.01$. Table 2 shows ACC based on combined data from FC and GB).

PF–L relationship estimated based on the autodiametric method (a = 0.05, b = 3.2901) did not differ significantly ($p > 0.05$) from those based on the gravimetric method (a = 0.03, b = 3.4272), that means any Greenland halibut fecundity estimation based on the autodiametric method can be compared with previous estimations based on the gravimetric method. All the results presented below are focused on autodiametric estimates. Mean PF (i.e. total number of oocytes per individual) is much lower for Greenland halibut in the GSL relative to the other areas examined (Table 3). However, these estimates are confounded by differences in fish length as fish sampled from the GSL were much smaller than those collected from other areas.

Examining PF in relation to fish length revealed a significant power relationship in all areas (Table 2). Comparison of PF–L relationships suggested no difference in fecundity-at-length among areas (Figure 4a). Low fecundities observed in the Gulf St Lawrence were simply due to the smaller size of fish.

Discussion

Greenland halibut have determinate fecundity but differ from those species for which the autodiametric method has been calibrated in that there is a potentially confounding second group of vitellogenic oocytes, developing for the subsequent year. This study demonstrates that, despite the unusual oocyte development strategy, ACCs can still be fitted to estimate fecundity of Greenland halibut based on the most advanced cohort of oocytes (G1).

Surprisingly however, Greenland halibut from the various areas examined here demonstrated spatial differences in ACCs, with packing density at a given oocyte size being significantly lower in the GSL than other areas and significantly higher in the SNF area than in the other areas. To our knowledge, this is the

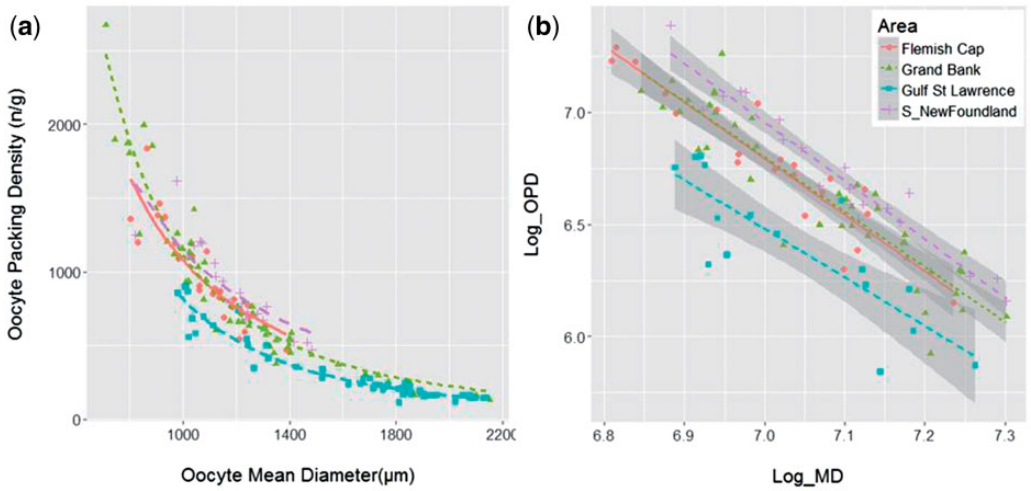

Figure 3. (a) Relationship between OPD and MD in each area; (b) relationship between log-transformed variables considering only the range between 900 and 1500 μm. Shaded area = 95% CIs.

Table 2. Parameters of ACCs and fecundity–length potential relationship for Greenland halibut from the different studied areas.

Area	Autodiametric curve				Fecundity-length			
	a	b	RSE	df	a	b	RSE	df
Flemish Cap	5.444×10^{8}	−1.901	163.5	22	0.1592	3.0310	25 470	21
GBs	1.032×10^{10}	−2.322	145.7	54	0.3648	2.8288	18 680	54
GSL	7.795×10^{9}	−2.327	60.6	53	0.0016	4.0960	3644	52
SNF	1.282×10^{8}	−1.683	156.4	19	0.0779	3.1999	6934	19
FC and GB	6.233×10^{9}	−2.251	154.4	78	–	–	–	–
All areas combined	–	–	–	–	0.0669	3.2223	15 460	152

a, intercept; b, slope; RSE, residual standard error; df, degrees of freedom.

Table 3. Range of values (average) of fish size and total fecundity, estimated based on the autodiametric method, in each study area.

Area	Fish size (cm)	Total fecundity
FC	60–98 (78.2)	34 598–232 079 (90 133)
GB	51–95 (72.0)	14 854–153 799 (67 778)
GSL	38–73 (48.3)	6062–64 822 (15 029)
SNF	60–88 (70.5)	35 731–121 632 (66 612)

first time that differences between autodiametric curves of the same species but from different areas have been reported (McElroy *et al.*, 2013). The reason(s) for these spatial differences are not certain but could be related to (i) the unusual oocyte development pattern, (ii) spatial differences in oocyte biochemistry, or (iii) differences in sampling methodology used by labs working in the respective areas.

In the first instance, it is possible that the spatial differences in ACCs are due to the complex nature of the oocyte development pattern for this species. Whether expressed as weight or volume fraction, the changes in gonad size that occur during development are attributed primarily to the extensive growth of vitellogenic oocytes. Other components of the ovaries, including immature oocytes, tunica, germinal epithelium, connective tissue, vascular system, glands, melanomacrophage bodies, atretic oocytes, etc. (Guraya, 1986, 1994; Kurita and Kjesbu, 2009 and references therein) are thought to have only a minimal influence (Kurita and Kjesbu, 2009; Schismenou *et al.*, 2012). Indeed, the relative contribution of vitellogenic oocytes gets even higher as vitellogenesis proceeds. Nevertheless, the relative contribution of other gonad components may depend on the oocyte recruitment strategy and may be more variable, e.g. in species with indeterminate fecundity (Kurita and Kjesbu, 2009; Korta *et al.*, 2010; Schismenou *et al.*, 2012). In Greenland halibut the presence of a second group of vitellogenic oocytes is a complicating factor in understanding the relative growth and contribution of various ovary components. Oocytes in the G1 group are much larger than those in the G2 group and should still have a much larger influence on packing density calculations than other ovarian components. In fact, G1 influence would be higher the more developed the gonad (i.e. the larger the G1 oocytes are); subsequently, ACCs differences between studied areas could be caused by spatial differences in maturity stage of the gonad (more or less advance in the spawning season). A protracted spawning season for Greenland halibut in the Northwest Atlantic has been reported (Fedorov, 1968; Junquera and Saborido-Rey, 1995), and although most of the gonad samples for the present study were taken in summer, the stage of maturation was not necessarily the same for all areas. The relative growth dynamics of G1 and G2 oocytes are not fully understood and any potential for spatial differences in this dynamic could lead to spatial differences in ACC such as those observed here. Given the particular oocyte dynamics of this species, more studies on the dynamics of G2 and successive cohorts should be carried out to robustly apply the Oocyte Packing Theory for estimating fecundity in Greenland halibut.

In addition to the relative numbers of the various oocyte classes, it is also possible that spatial differences in environmental conditions have influenced the actual biochemical composition (proteins, lipids, fatty acids, etc.) of those oocytes and hence influenced their size and weight. It is well known that oocyte composition varies in response to environment in order to guarantee egg development, buoyancy and survival (Nissling and Westin, 1991; Nissling *et al.*, 1994; Ospina-Álvarez *et al.*, 2012; Dominguez-Petit *et al.*, 2013). In fact, oocyte characteristics respond not only to maternal attributes (Vallin and Nissling, 2000),

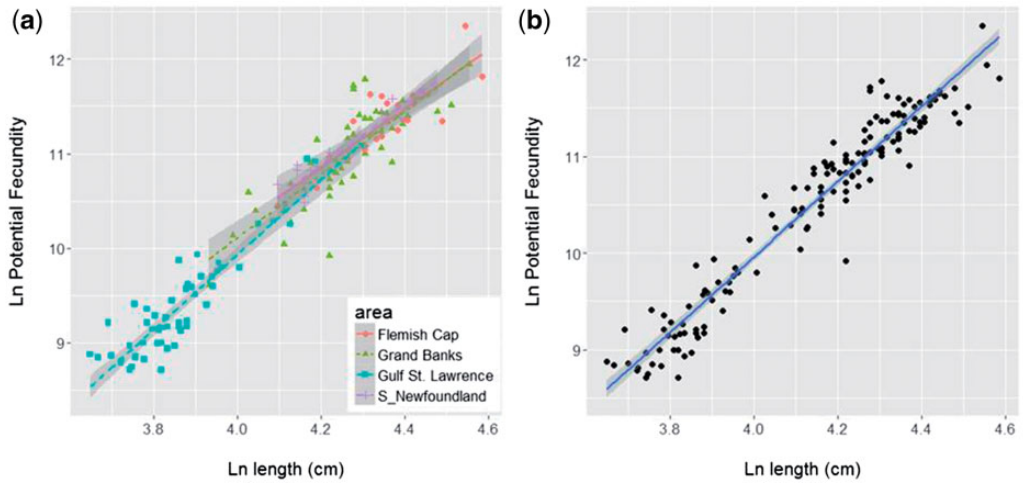

Figure 4. Relationship between log-transformed PF, estimated based on the autodiametric method, and length for (a) each studied area and (b) all areas combined. Shaded area = 95% CIs.

but also environmental factors like variations in water density (temperature and salinity), the quality of available prey to females and presence of pollutants or other metabolic stressors (Brooks *et al.*, 1997). Despite a large number of studies about environmental and maternal effects on egg quality, most of them are focused on egg size and morphology, but few of them pay attention on egg composition; Johnston and Leggett (2002) observed that the relationship between egg dry mass and maternal length and age varied among North American populations of walleye (*Stizostedion vitreum*), while Pickova *et al.* (1997) reported differences in fatty acid composition of eggs of Atlantic cod from the Baltic Sea and Skagerrak stocks, suggesting a strong influence of genetics on egg lipid composition. Although variability of egg composition among stocks of Greenland halibut has not been examined, studies on walleye and Atlantic cod support the hypothesis that differences in ACCs of Greenland halibut from the studied areas may result from differences in egg composition between stocks, which may be an adaptation to the characteristics of the spawning habitat.

It is also possible that the observed differences in ACCs are related to differences in sampling methodology. Although all labs used 4% buffered formaldehyde as fixative, there were differences in methodology that influenced the size of the sample being fixed and such differences could differentially influence oocyte sizes. NAFC used micropipettes to take a fresh sample of gonad that was then fixed while IML and IEO fixed a large portion or even a whole lobe of gonads to take fecundity subsample afterwards. Formalin-fixation results in an increase in oocyte size, with the influence of fixation on oocyte size increasing inversely to sample size (Klibansky and Juanes, 2008; Schismenou *et al.*, 2012). Hence the weight of micropipetted samples as well as diameter of large oocytes could be more affected by the fixative than the rest of samples. Fixed-fresh gonad weight relationship was calculated to minimize this effect, and no significant differences in results were observed when fix or fresh sample weight was used to estimate OPD. On the other hand, ACC based on samples taken in 3 L area by both IEO and NAFC with the two different methods were compared and no significant differences were observed ($p > 0.05$). Unfortunately, the whole range of sample weights was not available to estimate the influence of fixation depending on sampling procedure (micropipette vs. large portion), so the comparison was not as robust as desirable. However, the results of fixed-fresh gonad weight relationship and the comparison between ACCs from IEO and NAFC, indicate that spatial differences observed are mainly due to biological reasons rather than methodological differences. Nevertheless, the present results should be interpreted with caution because differences between ACCs from each area could be higher or lower than those reported in this study.

In the case of Greenland halibut, the previously described biases could be minimized by adjusting sampling protocols. For that purpose, we recommend (i) to select ovaries as close to the onset of the spawning season as possible, when the influence of pre- or early vitelogenic oocytes on volume fraction of advanced yolked oocytes is low (Kurita and Kjesbu, 2009) and (ii) fixing a large piece of ovary and taking the subsample for estimation of OPD afterwards (Klibansky and Juanes, 2008) to reduce the impact of fixation on both oocyte diameter and sample weight.

In general, compared with other species, Greenland halibut shows low OPD at MD (Figure 5), independently of the study area; more specifically at low MD (<1200 μm), the number of oocytes per gram of ovary in Greenland halibut is much lower than expected for some other species. For example, in an ovary with MD = 1000 μm, expected OPD in Greenland halibut would be between 800 and 1150 oocytes·g^{-1} while for the other species would be between 1540 oocytes·g^{-1} of European plaice and 1830 oocytes·g^{-1} of Atlantic herring (Figure 5). At larger oocyte MD (>1200 μm), the OPD of Greenland halibut is similar to that of another flatfish species, the European plaice. Differences in ACCs at smaller oocyte sizes are likely related to the particular oocyte dynamics of the species, more specifically to the volume fraction of G2 and previtelogenic cohorts. As oocyte maturation progresses, the volume fraction of G1 probably becomes so high that small oocytes volume have an insignificant effect, because of this no differences are found between Greenland halibut and European plaice OPD when oocytes are >1200 μm.

Despite differences on ACCs between areas, no differences in fecundity at length were observed, except for slightly lower values for specimens from GSL. Probably, these differences are due to differences in female size more than reproductive potential variations

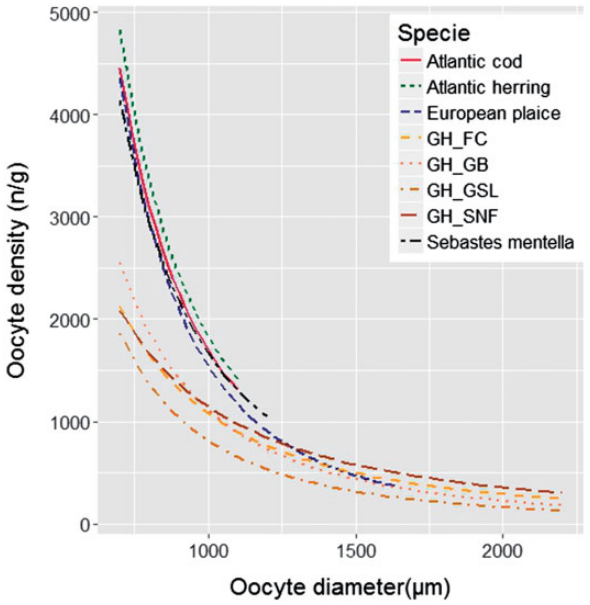

Figure 5. ACC of different species. GH, Greenland halibut; FC, Flemish Cap; GB, Grand Banks; GSL, Gulf St Lawrence; SNF, South Newfoundland. Calibration curves of Sebastes mentella, European plaice, Atlantic cod, and Atlantic herring were taken from Witthames *et al.* (2009).

because sampled females from GSL are significantly smaller than those from the other areas. Dwarfism has been recently reported in other species from the GSL as epigenetic adaptation to climate conditions of the habitat (Lighten *et al.*, 2016). These changes in gene expression probably affect not only growth but also other physiological aspects like maturation, aerobic capacity or even behaviour. No studies on the influence of female size on oocyte packing capacity have been carried out. Similarly, there are no studies on the effect that female physiology could have on the dynamics of G2 and successive cohorts in Greenland halibut. Investigation of these aspects could clarify the reasons behind differences of ACCs and fecundity in Greenland halibut from the studied areas.

The main conclusion of this study is that the autodiametric method can be applied to estimate fecundity in Greenland halibut, although the calibration curve should be stock-specific. The effect of the dynamics of G2 and successive cohorts on the volume fraction of G1 cohort and, subsequently, on the reliability of fecundity estimations is still unknown, and because of this, we recommend using samples with mean oocyte diameter >1200 µm in order to increase consistency of fecundity estimations and reduce bias. In any case, more research on the dynamics of all oocytes cohorts as well as factors (endo- or exogenous) determining oocyte packing capacity of females and potential habitat-adaptations of oocytes density would be necessary to understand geographical differences.

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