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

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13
14 **Running Title:** Autodiametric method applied to Greenland halibut fecundity

15
16 **Abstract**

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18 The autodiametric method is a highly streamlined method for estimating fecundity of fish with a
19 determinate oocyte development pattern. Greenland halibut is a deepwater flatfish presenting a very
20 peculiar reproductive strategy, with ovaries showing the simultaneous presence of both a cohort of
21 large vitellogenic oocytes (developing for the current year) and a cohort of small vitellogenic oocytes
22 (developing for the subsequent year). The first objective of this study was to determine if the
23 autodiametric method could be reliably used for estimating fecundity of Greenland halibut, and the
24 second one was to test spatial differences in the autodiametric calibration curve (ACC) and potential
25 fecundity in the Northwest Atlantic (Div. 3M, Div. 3LNO, Div. 3Ps and Div. 4S). Results showed
26 that the autodiametric method can be applied to estimate fecundity in Greenland halibut. Spatial
27 differences in ACCs (3M = 3LNO ≠ 3Ps ≠ 4S) were observed, but did not translate into differences
28 in fecundity at length. This is the first time that differences between ACCs of the same species but

from different areas have been reported. These differences could be the result of (1) the unusual oocyte development pattern, or (2) spatial differences in oocyte biochemistry, however interpretation is complicated by differences in sampling methodology between laboratories. More research on the relative dynamics of oocyte cohorts simultaneously present in Greenland halibut ovaries and the factors (endo- or exogenous) that influence oocyte packing density could lead to a better understanding of the observed geographical differences.

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

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 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. Mean oocyte diameter of an ovarian subsample is quickly obtained using an automated image analysis system and then converted to oocyte packing density, 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; Kennedy et al. 2008; Boulcott and Wright 2008; Lambert 2008; Witthames et al. 2009; Alonso-Fernández 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. . 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 considered separate from the much larger stock off the east coast (Arthur and Albert, 1993) and the northern stock between Canada and Greenland. 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 greater than one

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 (Junquera et al., 1999), the Gulf of St. Lawrence (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 oocyte packing density 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.

113 presence of a confounding secondary group of vitellogenic oocytes). Second, autodiametric
114 calibration curves (ACC) were developed and compared for fish collected from four general areas in
115 the Northwest Atlantic: Flemish Cap (NAFO Div. 3M), Grand Bank (NAFO Divs. 3LNO), Southern
116 Newfoundland (NAFO Subdiv. 3Ps) and the Gulf of St. Lawrence (NAFO Div. 4S). Finally, these
117 ACC were used to estimate potential fecundity of Greenland halibut from the various areas and test
118 for regional differences.

119

120 **Material and Methods**

121

122 This study brings together work done by three different laboratories to examine fecundity of
123 Greenland halibut from four different areas in the Northwest Atlantic. Data from the Gulf of St.
124 Lawrence were collected by the Institute Maurice Lamontagne (IML, DFO, Canada). Data from
125 southern Newfoundland (NAFO Subdiv. 3Ps) were collected by the Northwest Atlantic Fisheries
126 Centre (NAFC, DFO, Canada). Data from the Flemish Cap (Div. 3M) were collected by the
127 Oceanographic Centre of Vigo (IEO, Spain) and data from the eastern edge of the Grand Bank
128 (Divs. 3LNO) were collected by both NAFC and IEO.

129

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

141 commercial surveys and had fork lengths of 51-95 cm. Fish from the Flemish Cap (3M) were
142 collected in June-August 2004-2008 from scientific surveys (fork lengths of 60-98 cm) and in April
143 2007 and 2008 from commercial surveys (fork lengths of 58-89 cm).

144

145 Ovaries to be used for fecundity analyses were either fixed onboard the vessel (IML, IEO) or were
146 left in the gutted fish carcass (0-2 days) on ice until landing (NAFC). For ovaries fixed at sea,
147 subsamples were taken by teasing out a small piece of tissue using pointed forceps, while for the
148 non-fixed ovaries; subsamples of fresh tissue were collected with a positive displacement pipette and
149 subsequently fixed. Table 1 summarizes sampling procedures in each lab. The fixative used by all
150 three laboratories was 4% phosphate buffered formaldehyde. Care was taken to avoid collecting
151 ovarian wall tissue as part of the subsamples. The gonad subsample weight varied between 0.03 and
152 3.4 g depending on the method used for subsampling and the developmental stage of the ovaries:
153 more tissue was needed from ovaries with larger oocytes in order to get a sufficient number of
154 oocytes to be representative of the whole gonad (Gundersen and Emblem, 2002). Oocyte counts and
155 sizes were obtained by examining the ovarian subsample whole-mounts via an image analysis
156 system and the freeware ImageJ or software Leica QWin, depending on the lab.

157

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

166

167 The unusual oocyte development strategy employed by Greenland halibut poses challenges for
168 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 potential fecundity 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 700-1000 µm represented the most advanced oocytes in the ovaries, G1 was not clearly separated from G2, and all oocytes larger than 700 µm were counted and measured to estimate ACC. No G2 oocytes were counted if G1 oocytes were present (i.e. only the most advance oocytes were counted).

192

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

195

$$OPD = a \cdot MD^b$$

197

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

200

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

211

$$212 \quad r^2 = 1 - (\text{Residual Deviance} / \text{Null Deviance}).$$

213

214 The potential fecundity for each fish was estimated based on oocyte density (oocytes $\cdot \text{g}^{-1}$) and the
215 total weight of the ovaries. A potential fecundity-length (PF-L) relationship was estimated in each
216 area and regional differences were tested by fitting data to the GLM $\text{PF} \sim \text{L} + \text{Area}$. Goodness of fit of
217 the model was calculated as explained above. Tukey's range test was used for *post hoc* pair-wise
218 comparisons. In all analyses, differences were considered statistically significant when the p-value
219 was lower than 0.01.

220

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

225

226 **Results**

227

228 The observed range of MD was variable among areas, ranging from 803-1,388 μm for the Flemish
229 Cap, 710-2,153 μm for the Grand Bank, 981-2,143 μm in the Gulf St. Lawrence, and 820-1,483 μm
230 South of Newfoundland. Observed OPD ranges were 469-1,840 oocytes·g⁻¹, 129-2,673 oocytes·g⁻¹,
231 113-900 oocytes·g⁻¹ and 473-1,616 oocytes·g⁻¹ for FC, GB, GSL and SNF respectively.

232

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

237

238 Regional differences in ACCs were statistically significant. This was true regardless of whether
239 comparisons were based on the full range of observed oocyte diameters ($r^2=0.96$, AIC: -147.8,
240 $df=151$. Figure 3a) or only the overlapping range ($r^2=0.88$, AIC: -115.3, $df=93$) (Figure 3b). *Post hoc*
241 analysis showed no significant difference in ACCs between the Flemish Cap and Grand Bank
242 ($p>0.05$). However, these two areas were statistically different from the Gulf of St. Lawrence
243 ($p<0.01$) and Southern Newfoundland ($p<0.01$) and those two areas differed from each other as well
244 ($p<0.01$. Table 2 shows ACC based on combined data from Flemish Cap and Grand Bank).

245

246 Potential fecundity-length relationship estimated based on the autodiametric method ($a=0.05$,
247 $b=3.2901$) did not differ significantly ($p>0.05$) from those based on the gravimetric method ($a=0.03$,
248 $b= 3.4272$), that means any Greenland halibut fecundity estimation based on the autodiametric
249 method can be compared with previous estimations based on the gravimetric method. All the results
250 presented below are focused on autodiametric estimates. Mean potential fecundity (i.e. total number
251 of oocytes per individual) is much lower for Greenland halibut in the Gulf of St. Lawrence relative
252 to the other areas examined (Table 3). However, these estimates are confounded by differences in

fish length as fish sampled from the Gulf of St. Lawrence were much smaller than those collected from other areas.

255

Examining potential fecundity in relation to fish length revealed a significant power relationship in all areas (Table 2). Comparison of potential fecundity - length 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.

260

261 Discussion

262

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, autodiametric calibration curves can still be fitted to estimate fecundity of Greenland halibut based on the most advanced cohort of oocytes (G1).

268

Surprisingly however, Greenland halibut from the various areas examined here demonstrated spatial differences in autodiametric calibration curves, with packing density at a given oocyte size being significantly lower in the Gulf of St. Lawrence (GSL) than other areas and significantly higher in the southern Newfoundland (SNF) area than in the other areas. To our knowledge, this is the 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 (1) the unusual oocyte development pattern, (2) spatial differences in oocyte biochemistry, or (3) differences in sampling methodology used by labs working in the respective areas.

278

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; Guraya 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, for example, 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.

304

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), 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 3L area by both IEO and NAFC with the two different methods were compared

337 and no significant differences were observed ($p>0.05$). Unfortunately, the whole range of sample
338 weights was not available to estimate the influence of fixation depending on sampling procedure
339 (micropipette *versus* large portion), so the comparison was not as robust as desirable. However, the
340 results of fixed-fresh gonad weight relationship and the comparison between ACCs from IEO and
341 NAFC, indicate that spatial differences observed are mainly due to biological reasons rather than
342 methodological differences. Nevertheless, the present results should be interpreted with caution
343 because differences between ACCs from each area could be higher or lower than those reported in
344 this study.

345

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

352

353 In general, compared to other species, Greenland halibut shows low OPD at MD (Figure 6),
354 independently of the study area; more specifically at low MD ($<1200\ \mu\text{m}$), the number of oocytes
355 per gram of ovary in Greenland halibut is much lower than expected for some other species. For
356 example, in an ovary with MD equal to $1000\ \mu\text{m}$, expected OPD in Greenland halibut would be
357 between 800 and $1150\ \text{oocytes}\cdot\text{g}^{-1}$ while for the other species would be between $1540\ \text{oocytes}\cdot\text{g}^{-1}$ of
358 European plaice and $1830\ \text{oocytes}\cdot\text{g}^{-1}$ of Atlantic herring (Figure 5). At larger oocyte MD (>1200
359 μm), the OPD of Greenland halibut is similar to that of another flatfish species, the European plaice.
360 Differences in ACCs at smaller oocyte sizes are likely related to the particular oocyte dynamics of
361 the species, more specifically to the volume fraction of G2 and previtelogenic cohorts. As oocyte
362 maturation progresses, the volume fraction of G1 probably becomes so high that small oocytes
363 volume have an insignificant effect, because of this no differences are found between Greenland
364 halibut and European plaice OPD when oocytes are larger than $1200\ \mu\text{m}$.

365

366 Despite differences on ACCs between areas, no differences in fecundity at length were observed,
367 except for slightly lower values for specimens from GSL. Probably, these differences are due to
368 differences in female size more than reproductive potential variations because sampled females from
369 GSL are significantly smaller than those from the other areas. Dwarfism has been recently reported
370 in other species from the Gulf of St. Lawrence as epigenetic adaptation to climate conditions of the
371 habitat (Lighten *et al.*, 2016). These changes in gene expression probably affect not only growth but
372 also other physiological aspects like maturation, aerobic capacity or even behaviour. No studies on
373 the influence of female size on oocyte packing capacity have been carried out. Similarly, there are no
374 studies on the effect that female physiology could have on the dynamics of G2 and successive
375 cohorts in Greenland halibut. Investigation of these aspects could clarify the reasons behind
376 differences of ACCs and fecundity in Greenland halibut from the studied areas.

377

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

387

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392

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Table 1. Summary of sampling procedure in each laboratory. 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

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

Table 2. Parameters of autodiametric calibration curves and fecundity-length potential

relationship for Greenland halibut from the different studied areas. a: intercept, b: slope, RSE: residual standard error, df: degrees of freedom.

Area	Autodiametric curve				Fecundity-length			
	a	b	RSE	df	a	b	RSE	df
Flemish Cap	$5.444 \cdot 10^8$	-1.901	163.5	22	0.1592	3.0310	25470	21
Grand Banks	$1.032 \cdot 10^{10}$	-2.322	145.7	54	0.3648	2.8288	18680	54
Gulf St. Lawrence	$7.795 \cdot 10^9$	-2.327	60.6	53	0.0016	4.0960	3644	52
S Newfoundland	$1.282 \cdot 10^8$	-1.683	156.4	19	0.0779	3.1999	6934	19
Flemish C. & G.Banks	$6.233 \cdot 10^9$	-2.251	154.4	78	-	-	-	-
All areas combined	-	-	-	-	0.0669	3.2223	15460	152

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
Flemish Cap	60-98 (78.2)	34,598-232,079 (90,133)
Grand Banks	51-95 (72.0)	14,854-153,799 (67,778)
Gulf St. Lawrence	38-73 (48.3)	6,062-64,822 (15,029)
S Newfoundland	60-88 (70.5)	35,731-121,632 (66,612)

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

270x188mm (300 x 300 DPI)

Figure 2. Whole mount gonad sample. G1: cohort to be released in the current spawning season ($>1000\ \mu\text{m}$) and G2: cohort to be released in the next spawning season ($< 1000\ \mu\text{m}$). Oocytes between 700 and $1000\ \mu\text{m}$ are opaque and were also included in the autodiametric calibration curve when G1 oocytes were not evidently present.

127x95mm (300 x 300 DPI)

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

135x65mm (300 x 300 DPI)

Figure 4. Relationship between log-transformed potential fecundity, estimated based on the autodiometric method, and length for a) each studied area and b) all areas combined. Shaded area = 95% confidence intervals.

134x65mm (300 x 300 DPI)

Figure 5. Autodiametric calibration curve 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).

141x146mm (300 x 300 DPI)