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## The fate of vitellogenic follicles in experimentally monitored Atlantic cod *Gadus morhua* (L.): application to stock assessment

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**Abstract**

In this paper we report on the fate of vitellogenic follicles (VF) as either alpha atretic follicles (αF) or post-ovulatory follicles (POFs) using histology and captive Atlantic cod (*Gadus morhua*) in three experiments.

   In Experiment 1 the production and persistence of αF was determined by taking repeated biopsy samples from tagged females held in temperature regimes (mean ± SD) controlled at 4.5 (0.3) and 8.1 (0.3) $^{\circ}$C. The αF lasted (mean ± 2 SE, n) 5.3 days (2.5, 8] and 9.7 days (4.9, 8) in the warmer and cooler water respectively and the combined average was 7.5 days (2.9, 16).

26    In Experiment 2 we took biopsy samples at intervals and monitored egg production from

27    individual females accompanied by a male and used the stage of egg development to age POFs

28    found in the biopsy samples. The females, some immature, were killed at intervals, up to 45 days

29    post spawning, and then the biopsy and ovary samples were stained by periodic acid Schiffs'

30    reagent to prepare descriptions of POFs aged from 11 hours to 45 days old. Spent female ovaries

31    contained POFs, and a thicker ovarian wall (tunica) exceeding 0.34 mm whilst immature fish lacked

32    POFs and their ovary tunica was thinner (less than 0.15mm). In Experiment 3 the persistence of

33    POFs was monitored in a simulated North Sea (10-16.1 $^{\circ}$C) and Barents Sea (7.5-11.2$^{\circ}$C) regime

34    using ovary sections stained by periodic acid Schiffs' reagent. In both regimes the POFs regressed

35    at a temperature sensitive rate during the experiment lasting 104 days. Some αF from large VF

36    persisted longer than expected (more than four months after spawning) and were called cysts based

37    on their appearance and greater expected lifetime.  These histological characteristics were

38    successfully applied to assess maturity of wild cod caught on surveys in the North and Barents Seas

39    after an assumed 150 and 310 days respectively after the spawning season. Taken together this

40    article presents reliable figures on the lifetime of atretic and post-ovulatory follicles as well as

41    variation in ovarian thickness with spawning experience, which will be most useful input in the

42    further work to assess reproductive potential.

43

44    **1. Introduction**
45

46    Female reproductive potential plays a pivotal role in the capacity of wild fish populations to

47    sustain their numbers when facing heavy fishing mortality so it is important to establish the

48    dynamics of egg production. Although Virtual Population Analysis (VPA)  makes it possible to

49    assess numbers by age class (Beverton and Holt, 1957)  it is also important that we assess the

50    relationship between stock and reproductive potential (Murawski et al., 2001;Witthames and

51    Marshall, 2008). In such assessment it is also important to identify the spawning stock from the

52    immature component (Hunter and Macewicz, 2003), especially when the stock is dominated by

53    small young fish after high fishing mortality. In the case of Atlantic cod (*Gadus morhua*) the

54    external morphology of the ovary has been linked to a histological description of females caught

55    during the spawning season (Morrison, 1990; Burton et al., 1997) to classify individuals as

56    immature and mature. It is also needed to develop criteria to assess maturity outside the spawning

57    season either because the population is less clustered or to fit in with other survey commitments.

58    Based on previous reports postovulatory follicles [POFs (Saborido-Rey and Junqera, 1998; Rideout

59    et al., 2005)] or ovary wall (tunica) thickness (Burton et al., 1997) are possible markers of past

60    spawning activity in cod but more experimental validation is required. One experiment (Burton et

61    al., 1997) did compare ovary tunica thickness in immature and maturing female cod but no data was

62    provided on POFs. We felt it important to revisit these studies using new experimental procedures

63    to track identified females in order to develop maturity assessment criteria that are more objective

64    and less susceptible to qualitative judgement (Hunter and Macewicz, 2003).

65        The annual egg production method [AEPM (Lockwood et al., 1981)] is an alternative to VPA as

66    it is a fisheries-independent method that can be applied when the fishery is closed to allow stock

67    recovery. In a recent application of this method it was reported that cod, sole (*Solea solea*) and

68    plaice (*Pleuronectes platessa*) spawning stock biomass (SSB) was 2.3, 2.7 and 4.3 times, greater

69    compared to VPA results (Armstrong et al., 2001). During the course of this type of assessment it

70    became clear that not all yolk follicles, comprising the potential fecundity ($F_p$), expressed relative to

71    body weight [($F_{pr}$) gram$^{-1}$ (g$^{-1}$)], complete the growth phase (vitellogenesis) during maturation and

72    abort their development through atresia down regulation (Kurita et al., 2003; Thorsen et al.; 2006;

73    Kennedy et al., 2007; Witthames et al., 2009). Loss of $F_{pr}$ prior to spawning can be accounted for by

74    selecting only pre-spawning females in late maturity (Witthames et al., 2009)  but further atresia

75    may also occur after the start of spawning (Kjesbu et al., 1991; Rideout et al., 2005; Kraus et al.,

76    2008). Atresia during spawning would therefore directly increase the estimated spawning stock

77     biomass ($B_s$) by reducing the individual relative realised fecundity ($F_r$ $g^{-1}$ total fish weight) and

78     should be included in the AEPM equations:

79                                  $$B_s = \frac{TEP}{Fr} \qquad\qquad (1)$$

80     where TEP = population total egg production and

81                           $$F_r = F_{pr} - Fpop_\alpha \qquad\qquad (2)$$

82     where $Fpop_\alpha$ is the geometric mean of alpha atretic follicles $g^{-1}$ total fish weight in the population

83     excluding fish with no atresia (Hunter and Macewicz, 1985a).  A geometric mean is used because

84     $Fpop_\alpha$ has a log normal distribution and is calculated using Equation 3:

85                   $$Fpop_\alpha = F_{pr} * \alpha Fpop * \frac{Sp}{D} * P \qquad\qquad (3)$$

86     where D is the number of days alpha atretic follicles take to regress to the beta stage, $\alpha Fpop$ the

87     population average of the proportion of yolk follicles in the alpha atretic stage ($\alpha F$),  Sp (days)

88     spawning duration (Kjesbu et al., 1991; Horwood, 1993), and P is the proportion of females in the

89     population containing $\alpha F$. The value of P adjusts $Fpop_\alpha$ down to correct for the proportion of fish

90     with no atresia (Armstrong et al., 2001). Although the atretic loss can approach a significant part of

91     the $F_{pr}$, the experimental basis to determine D is not well understood. Only two tank experiments

92     (Hunter and Macewicz; 1985a, Kjesbu et al., 1991) and one on wild Atlantic herring (*Clupea*

93     *harengus*) populations (Kurita et al., 2003) have provided any specific information on the dynamics

94     of the process. A further uncertainty is the influence of temperature on the rate of follicle regression

95     and this has also not been investigated. Published results show some consistency but there is a clear

96     need to determine how long the $\alpha F$ stage, defined in Hunter and Macewicz (1985a ) and Kjesbu et

97     al. (1991), persists, especially the corresponding error terms, and the consequences of this variation

98     for the estimation of realised fecundity (Óskarsson et al., 2002).

99       This paper details three experiments to investigate the fate of vitellogenic follicles in captive

100    Atlantic cod by accounting for their $F_p$ as either egg production ($F_r$) or follicular atresia. We use the

101 term follicle referring to both the oocyte and outer follicle layers (Tyler and Sumpter, 1996).  In

102 Experiment 1 we assessed atretic vitellogenic follicle production by studying changes in the ratio of

103 normal to alpha, and a combined beta and gamma stage using published criteria (Hunter and

104 Macewicz, 1985a).  We exposed the fish to temperatures considered typical of those experienced by

105 North Sea and Barents Sea cod stocks so that the results would be relevant to a range of habitat

106 occupied by this species. Experiment 2 monitored egg production, and POF regression in mature

107 fish. The ovaries from immature and mature females, known to have spawned, were compared in

108 relation to ovary tunica thickness, residual $\alpha F$, atretic follicles and POFs. In Experiment 3 the

109 persistence of POFs was studied simulating a Barents Sea and North Sea spring warming cycle

110 from the end of April to August.  Consideration was then given to using the above spawning

111 markers to identify spent mature and immature wild cod collected 6 (North Sea) and 11 months

112 (Barents Sea) after the previous spawning season.

113 **2. Materials and methods**

114 *2.1. Experiment 1: $\alpha F$ production and fate*

115

116    Fish were sedated in 5 mg $l^{-1}$ metomidate dissolved in oxygenated sea water (Mattson and Riple,

117 1989) during all the handling and measurement operations in the experiment (Table 1). Prior to

118 starting the experiment a PIT tag (Destron Fearing, USA), was inserted subcutaneously into each

119 fish for subsequent identification and a biopsy sample was removed using a Pipelle de Cornier®

120 [Prodimed, Neuilly En Thelle, Picardie, France (Witthames et al., 2009)], from the ovary by

121 catheterisation through the genital pore (McEvoy, 1985; Kjesbu, 1989). The total mass (g) and total

122 length (cm) of each fish were also measured in this preparatory work. Each biopsy sample was

123 fixed in 3.6 % formaldehyde solution buffered to pH 7.0 by 0.1 M sodium phosphate (NBF) for a

124 minimum of two weeks before further processing. To identify and select only maturing fish  for the

125 experiment the leading follicle cohort (LC), defined as the average of the largest 10% of follicles,

126    was measured in a sample of 200 from the biopsy by image analysis (Thorsen and Kjesbu, 2001),

127    selecting females with developing oocytes, i.e., LC > 250 μm. Each tank was continually filled

128    (Kjesbu 1989) by ambient sea water (8.1 SD $0.3^{\circ}$C) until the experiment started (Table 1) and all

129    feeding stopped. At the start of the experiment the fish were divided between each tank after

130    removing a biopsy sample and the water temperature was either cooled or remained at ambient

131    (Table 1). Further biopsy samples were removed at regular intervals to monitor αF production (Fig.

132    1). All of the fish were killed by a standard procedure at the end of the experiment, after exposure to

133    a lethal dose of anaesthetic followed by severing the brain from the spinal chord.

134    Processing biopsy samples involved dehydration and embedding in Technovit resin (Tamro

135    Mikroskopi, Norway) to prepare 5 μm sections that were stained by periodic acid Schiff's (PAS)

136    and Mallory trichrome (Witthames and Greer Walker, 1995).  Follicles were classified (Fig 2) as

137    normal vitellogenic follicles (VF), alpha atretic follicles (αF) or a combined beta (βF) and gamma

138    follicles (γF) stage (Hunter and Macewicz, 1985a) since the β and (γF) stages were considered too

139    similar to be consistently scored separately (Ganias et al., 2008).  Three replicate samples,

140    averaging 168 (minimum 151 maximum 211) follicles, were scored in the first biopsy to determine

141    the proportion of each atresia class at the start of the experiment.  For each fish 2SE was added to

142    the mean value αF or a combined βF + γF (the reference level) so that if the reference level was

143    exceeded it would indicate new atresia production. Fish that contained no αF or βF + γ F in the first

144    biopsy were assigned a reference level based on the mean + 2SE of all the other reference values. In

145    each subsequent biopsy a further average of 165 (minimum 85 maximum 229) follicles was scored

146    in order to determine the production of each atretic class. The day of new production for αF and βF

147    + γ F was identified when the reference level was exceeded in a subsequent biopsy sample (Fig. 1).

148

149    *2.2  Experiment 2: POF production and a comparison of spent and immature ovaries*

150

151     Preparation of fish for the experiment (Table 1) followed the procedure detailed in Experiment 1.

152     Prior to the start of the experiment the fish were fed on moderate rations (Kjesbu et al., 1991) and

153     transferred to the experimental tanks when feeding was discontinued to monitor egg production

154     (Kjesbu 1989).

155     At the start of the experiment a biopsy was taken following brief sedation, as in Experiment 1,

156     and examined to determine sex and maturity status for selection of females used in the study (Table

157     1). Processing of biopsy samples followed the same protocol as Experiment 1. POFs were identified

158     using criteria for multiple spawning fish (Hunter and Macewicz, 1985b) and specifically for cod

159     (Murua et al., 2003) applied to PAS stained sections (Fig. 3).

160     Further biopsy samples were removed at intervals (Fig. 4) whilst egg production from each

161     female was monitored so that we could link POF persistence and morphology with a known

162     spawning history. Monitoring egg production involved estimating the number of eggs in each batch,

163     $F_r$ and the time of spawning based on temperature-specific egg development rates (Table 2) using

164     published data and methods (Kjesbu, 1989).

165     The experiment was terminated (Table 1) to remove the ovaries which were fixed for a minimum

166     of two weeks prior to cutting out whole cross sections 5 mm thick mid way from one end. Each

167     cross section was processed as the biopsy samples, in order to estimate the residual VF, and αF by a

168     stereometric method (Emerson et al., 1990).  Measurements of the ovary tunica thickness,

169     maximum previtellogenic oocyte diameter (repeated in seven microscopic fields) were made using

170     Myrmica 4 software with a resolution of 3.5 μm per pixel in each case.

171

172     *2.3  Experiment 3: fate of postovulatory and residual vitellogenic follicles*

173

174     This experiment was started (Table 1) in spring by killing five females,

175     using the same procedure as in Experiment 2, from a group that had just completed the annual

176    spawning cycle. This group was then divided between two tanks where the temperature was

177    controlled to simulate a North Sea or Barents Sea spring to summer warming regime (Fig. 5). Fish

178    were fed from the start of the experiment to satiation twice weekly until the experiment finished in

179    late summer after 104 days.  Further samples of five fish from both tanks (Fig. 5) were killed at

180    intervals until the end of the experiment. Each ovary sample was processed as the biopsy samples in

181    Experiment 2, to prepare stained histological slides to determine the rate of POF regression and to

182    look for the presence of residual vitellogenic follicles (together referred to as spawning markers).

183        All POFs encountered whilst scanning across the section were measured using a polygon

184    function (Myrmica 4 freeware [myrmica.co.uk]) to define the cross section area, until 20

185    observations were in the data set. The mean size of the largest two POFs from each sample was

186    taken as the leading POF cohort and assumed to originate from the last ovulation. The rate of POF

187    regression was investigated using an exponential decay model:

188                $y = a * \exp(-b * day)$                    (4)

189

190    where y = POF area and we test whether the same or area specific coefficients are required for the

191    Barents Sea and North Sea data to give the best fit.

192

193    *2.4. Spent-recovering wild fish ovary histology*

194

195        Cod were taken from trawl hauls made during the 'International bottom trawl survey' (IBTS) in

196    the third quarter from the North Sea and during the 'winter survey' in the first quarter from the

197    Barents Sea (Table 3). In each case the ovary was removed and a whole or part cross section was

198    fixed in NBF. The fixed tissue was processed into stained slides as above. These slides were

199    examined for the presence of POFs, residual atretic vitellogenic follicles (cysts) assumed to have

200    originated from the last spawning which occurred approximately 150 and 305 days previously in the

201    Northern North Sea and off the Lofoten Isles respectively. The ovary tunica thickness, when present

202    in the sample, and the cross section area of POFs was measured as in Experiment 2 and 3

203    respectively.

204

205    **3. Results**

206    *3.1 Experiment 1: αF production and fate*

207    The use of PAS Mallory to stain biopsies made it easy to visualise the transition of VF to αF

208    based on the fragmentation of the chorion and dissolution of the yolk (Fig. 2). Although the PAS

209    positive basement membrane was visible between the thecal and granulosa layers throughout

210    regression of VF to βF + γ F, it never became enlarged or pronounced as in older POFs. Vacuoles

211    and intercellular cavities were apparent in the βF + γ F stage but were spread out and small

212    compared to the large unstained lumen making up the central part of the POF (Figs. 2 and 3).

213    Only 8 of the 25 fish in each temperature regime (Table 1) produced αF and then βF + γ F to

214    exceed the αF and βF + γ F reference levels (Fig. 1). The αF stage was approximately twice as

215    abundant compared to the βF + γ F stage in both regimes but there was also considerable variation

216    in consecutive biopsy samples. There was an insignificant statistical effect ($P=0.125$) of

217    temperature on the mean duration (days) of αF, for all fish in the group although it was longer in the

218    cooler water 9.7 days [2 standard error (2 SE) 4.9] compared to 5.3 days (2 SE 2.5). The combined

219    data from each temperature regime gave an αF duration of 7.5 days (2 SE 2.9).

220

221    *3.2. Experiment 2: POF production and a comparison of spent and immature ovaries*

222

223    The two females Mat 1 and Mat 2 produced mostly 100% fertile regular batches of eggs,

224    spawning for the first time on the 22 February and 4 March respectively, whilst female Mat 3

225 produced a small batch on the 24 February before more regular batch production from 21 March

226 (Fig. 4). Biopsy samples taken prior to spawning, mostly from Mat 1, contained no POF like

227 structures but POFs appeared in all biopsies with increasing abundance following the start of

228 spawning. The POFs found in the first biopsy from Mat 1, were aged between 10.2 to 12.45 hours

229 old because the eggs at 32 blastomere stage originated from the first ovulation.

230     Thus our collection of biopsy samples and whole ovary sections were taken from 10 to 12.45

231 hours post spawning until 45 days after spawning had finished (Fig. 4). The POF aged at 10 to

232 12.45 hours old had collapsed to a thin curly band of granulosa and thecal cells lying each side of a

233 PAS stained basal membrane around a large lumen typically 530 µm across its longest axis (Fig. 3).

234 In Mat 3, killed just before spawning had finished (Fig. 4), there was a range of POF structures

235 originating from the regular succession of egg batches produced during the experiment. The largest

236 POF appeared similar to the example found in the first biopsy after spawning from Mat 2, but others

237 showed a gradation of size. Because we found that POFs persisted for at least 45 days post

238 spawning in the spent ovary of Mat 1 the range of POF structure in Mat 3 show the accumulation

239 over all the preceding spawning events for this fish. The smallest POF still showed pronounced

240 PAS staining of the residual basement membrane and a clearly defined central lumen.

241     Comparing ovaries from near spent or spent females (Mat 2 and 3) with immature females it was

242 noted that larger previtellogenic follicles were present in the immature fish (up to 185 (2SE 6) and

243 224 (2SE 12) µm, respectively) compared to 131 (2SE 18) µm in the two spent fish (Fig. 3). The

244 ovary tunica was much less developed, 120 µm thick, in the immature fish and up to 650 µm thick

245 in the ovary of Mat 2. Also in Mat 2 large atretic vitellogenic follicles were aggregated into a mass

246 in some cases so that it was difficult to see the boundary of each follicle.

247

248 *3.3. Experiment 3: fate of postovulatory and residual vitellogenic follicles*

249

250     The temperature regimes imposed in the tank water, simulating the Barents Sea and North Sea

251    spring summer warming regime, differed by 2.6$^{o}$C at the start of the experiment (Table 1) and

252    diverged to 4.9 $^{o}$C, (based on a 10 day moving average) when the final sample was taken 104 days

253    later (Fig. 5). POF shrinkage rates were significantly different in the two temperature regimes

254    (Table 4) so that the distribution of POF areas (Fig. 6) became marginally significantly different

255    after 104 days (t= 1.973, degrees of freedom = 5.56, P=0.0998 two sample Welch two sample

256    student t test). In each case the lumen of the POF was evident throughout regression whilst the area

257    of PAS staining was pronounced at first but became progressively reduced though still visible when

258    the last sample was taken in August (Fig. 3). Surprisingly, atretic follicles, referred to as cysts, were

259    still seen in some of the ovary sections taken in August from both temperature regimes. The

260    follicles concerned showed a thickened chorion, and occasionally, some yolk granules. The outer

261    follicle layers were fibrous with unstained void areas (Fig. 3).

262

263    *3.4. Spent-recovering wild fish ovary histology*

264

265     Extrapolating the separate temperature POF regression models (Table 4) to the number of days

266    post spawning, assumed 150 and 305 days after sampling for the Barents Sea and North Sea

267    respectively, suggested that POFs should still be visible. This was verified by a comparison of the

268    predicted and observed POF area (Fig. 6) with the latter being above or within the 95% confidence

269    interval of the prediction. Also seen in spent ovaries were large follicle cysts and thickened tunica

270    (Fig. 3) that were very similar in appearance when compared to spent females in Experiment 2.

271    Mostly the cysts were discrete objects in the cross section but in some cases cysts in close proximity

272    were aggregated into a mass where it was not possible to discern boundaries. Based on the presence

273    or absence of these spawning markers it was possible to distinguish between immature or post

274    spawning ovaries (Table 3).

275

276  **4. Discussion**

277

278      When we planned Experiment 1 there was little information on the temperature experienced by

279  free living Atlantic cod to decide on relevant experimental temperature regimes However, this

280  information is now gradually building up with the use of data storage tags in different waters (Godø

281  and Michalsen, 2000; Palsson and Thorsteinsson, 2003, Neat and Righton, 2007). These articles

282  show that the temperature range used in Experiment 1 were typical or slightly above temperatures

283  experienced by stocks, from the northern North Sea to north Iceland just prior to, or during

284  spawning (David Righton Cefas, UK, personal communication.).

285      Our estimated atretic follicle ($\alpha$F) duration would therefore be widely applicable although we

286  were disappointed by the low precision around the mean duration (D). Although data from wild

287  Atlantic cod populations show 1/3 of fish sampled  contain $\alpha$F (Armstrong et al., 2001; Kraus et al.,

288  2008; Witthames et al., 2009) we expected a higher proportions given the stress of the repeated

289  biopsy sampling. Higher levels of individual $\alpha$F and older atretic stages ($\beta$F+$\gamma$F) would be obtained

290  by an unbiased but much more laborious Disector method (Kjesbu et al. this monograph) but would

291  likely be of marginal interest in the present context. This approach was rejected because we were

292  concerned with relative changes of $\alpha$F and $\beta$F+$\gamma$F compared to normal vitellogenic follicles (VF).

293  Although we accept $\alpha$F and $\beta$F+$\gamma$F would be undersampled the error would be a constant bias rather

294  than subject to change during the short period of the experiment.

295      The production of $\alpha$F and $\beta$F + $\gamma$ F, in relation to the reference value, was similar though slightly

296  less in the case of $\beta$F + $\gamma$ F suggesting the $\beta$F + $\gamma$ F stage maybe shorter than that recorded for $\alpha$F.

297  Despite the effect of undersampling the $\beta$F + $\gamma$ F stage part of the explanation may be because the

298  most durable part of the follicle, the chorion, has disappeared by the end of the $\alpha$F stage, so there is

299  little solid material remaining to identify the final extinction phase of the follicle. However, an

300    alternative explanation has been reported in striped mullet [(*Mugil cephalus*) McDonough et al.

301    2005] and sardine [(*Sardinia pilchardus*) Ganias et al., 2007]: accumulation of $\beta F + \gamma F$ moves from

302    the epithelium and concentrates medially in the ovarian lamellae and therefore may be under

303    sampled by the biopsy pipelle.

304    The $\alpha F$ duration for each temperature regime determined from our experiments shows some

305    consistency compared with earlier reports, given the range of temperatures, maturity stages and

306    species (Table 5). It is likely that the rate of $\alpha F$ regression will follow the $Q_{10}$ rule (Schmidt-

307    Nielsen, 1978) so that its duration will be inversely proportional to water temperature but follicle

308    size, depending on maturity stage, will confound the effect of temperature. For example $\alpha F$ duration

309    in Atlantic herring varied between 5.8 days, during early ovary maturation of small follicles (500

310    µm) in July- October, to 9.1 days just prior to spawning when the follicles are approaching 1300

311    µm (Kurita et al., 2003. However, there is also inconsistency between the anchovy *Engraulis*

312    *mordax* rate [8 days at $16^{o}$C (Hunter and Macewicz 1985a)] where the developing follicles are

313    smaller compared to cod reported from 7.5 (our data) to 10 days (Kjesbu et al., 1991) at 4.5-9 ℃.

314    The persistence of regressing $\alpha F$ that were still present 150 days post spawning in both

315    experimental and wild fish was not expected based on all this evidence. We consider that these

316    structures should be more accurately referred to as cysts (Tomkiewicz et al., 2003) as they are not

317    following the normal dynamic of $\alpha F$ regression. Although our results confirm a recent study on

318    sardine (Ganias et al., 2008) that $\alpha F$ was a short term stage we believe that the largest vitellogenic

319    follicles, failing to enter final maturation, become encysted. In some cases we saw parts of cyst

320    aggregations resembling the much later delta stage of atresia (Hunter and Macewicz 1985a), i.e.,

321    without clearly defined boundaries between each follicle.

322    For the first time we report on changes in POF morphology and size from 12 hours after the first

323    spawning to 45 days post spawning in individual cod and over 104 days during the post spawning

324    period by sampling groups of cod. Although the data from the Barents and North Sea did not

325    separate completely during the experiment (P=.1) the distributions were moving apart and would

326    probably have separated if the experiment had lasted another 15 days. Our results support the

327    classical work describing the ageing process of POFs in captive anchovy (Hunter and Goldberg,

328    1980) and more recent studies in sardine where POF perimeter and shape were shown to shrink

329    rapidly (Ganias et al., 2007) but over a time scale measured in a few days. A 3D study on cod POF

330    shape (Korta et al., this monograph) also makes an interesting comparison. However, our

331    observation that POFs last months is quite different to the situation reported in anchovy (Hunter and

332    Macewicz 1985b) or sardine (Ganias et al., 2007). POFs in anchovy were thought to become very

333    reduced and difficult to distinguish from $\beta F$ or $\gamma F$ by the second day (Hunter and Macewicz

334    1985b). This may be more exaggerated if the ovary is fixed whole and subject to compression by

335    the ovary tunica rather than in small fragments (Witthames et al., 2009; Korta et al., this

336    monograph). In the case of cod we found the use of PAS stain and a central lumen that we followed

337    throughout POF regression made distinction between POF and $\beta F$ or $\gamma F$ unambiguous. The central

338    lumen was also considered an important criterion to distinguish POF from $\beta F$ or $\gamma F$ in the case of

339    sardine (Ganias et al., 2007). We also noticed that old POFs were very numerous and of similar size

340    and shape, whilst $\beta F + \gamma F$ were present in relatively low numbers and appeared with a less

341    convoluted outline compared to POFs.

342       Temperature has previously been shown to effect POF regression (Fitzhugh and Hettler, 1995;

343    Ganias et al., 2007) in warm-water species Atlantic menhaden (*Brevoortia tyrannus*) and sardine

344    living at 14.8 to 20$^{o}$C. The presence of POFs has, however, also been used to indicate previous

345    spawning events further away in time; in Flemish Cap cod POFs were stated to be present in the

346    ovaries 3-4 months after spawning (Saborido-Rey and Junqera, 1998). Our results agree with these

347    field results and provide a means to hindcast the time elapsed since spawning based on POF profile

348    area measured in section.

349     Comparing the temperature regimes we imposed during Experiment 3 it is now apparent that

350    both groups were exposed to warmer water than would be expected (Godø and Michalsen, 2000;

351    Neat and Righton, 2007). The North Sea regime was probably a few degrees higher than normal

352    during the post spawning season but in the summer more typical of the shallower Southern region

353    than the Northern North Sea. The Barents Sea simulation was probably several degrees warmer than

354    what would be expected when the fish move north into the Barents Sea after the spawning season..

355    However, based on the experimental data there should be no problem detecting POFs at least 150

356    days post spawning though in the more northerly cold areas this period could be extended, perhaps

357    to over a year. The POFs found in wild fish caught in the Northern North Sea (above $57^{\circ}$ North)

358    about 150 days post spawning, were mostly larger and outside the predicted confidence limits.

359    Temperature data from cod fitted with storage tags caught in the Northern North Sea (Neat and

360    Righton, 2007) show they live in colder water during the summer depending on locality [mean 7.6

361    (SD 1.86) -9.5 (SD 1.91) $^{\circ}$C] reflecting more closely the Barents Sea simulation. The situation is

362    further complicated because some cod frequent mostly shallower warmer water whilst others

363    occupy deeper colder offshore water during the summer even though they spawn in similar

364    temperature regimes [Icelandic cod: around $7^{\circ}$C (Palsson and Thorsteinsson, 2003)].

365    We have now applied the PAS stain to detect the presence of POFs in several species both

366    immediately post spawning and also after many months have elapsed (Skjæraasen et al., In press;

367    Witthames unpublished data). In a closely related gadoid, Atlantic haddock (*Melanogrammus*

368    *aeglefinus*), POFs were found in sections prepared from ovary samples taken in the third quarter

369    IBTS survey in the North Sea several months after their assumed last spawning season. In contrast

370    Scombroids such as Atlantic mackerel (*Scomber scombrus*) or Carangidae such as horse mackerel

371    (*Trachurus trachurus*) or Clupeids such as Atlantic herring produce POFs that do not stain as

372    effectively with PAS and appear to disappear within days, being absent in spent or partially spent

373    females. POFs in sardine also do not appear to persist over long periods and reach 0.010 mm in 3.5

374    days (Ganias et al., 2007) compared to about 50 and 100 days for the present North and Barents Sea

375    cod simulations respectively.

376        We see an important application of this work by providing experimental evidence to support

377    methodology to quantify the incidence of skipped spawning in cod population assessment (Rideout

378    et al., 2005; Skjæraasen et al., In press). The aim would be to classify the observed non-developing

379    fraction of females as i) immature, ii) mature spent and iii) skipped spawning  i.e. fish that spawned

380    in the previous year but are skipping the current spawning season. Important issues are the

381    persistence of spawning marker POFs, cysts and ovary tunica thickness in relation to the elapsed

382    time between the survey and the last or next spawning season. Our data would suggest that if POFs

383    are found and their size fits the regression path, taking into account the elapsed time between the

384    survey and the last spawning season, then this fish positively spawned during the previous season.

385    Further confirmation follows from the width of the ovary tunica and the presence of cysts, or

386    alternatively, if the tunica is less than 0.15 mm, then the female is immature. If the tunica is wider

387    than 0.15 mm and no POFs are present, although expected from the elapsed time since the last

388    spawning, then the female possibly skipped the last spawning. Experiment 2 however, would not

389    resolve whether a thickened tunica found in spent fish would persist if the fish skipped more than 2

390    years in succession. In cold water situations, like the Barents Sea where POFs appear to persist well

391    beyond the start of fecundity recruitment, lack of developing fecundity during the maturation season

392    combined with the presence of POFs indicate that the next spawning will be skipped. Although the

393    costs of the histology may prohibit its use on routine surveys it could be used as a quality assurance

394    tool for macroscopic maturity evaluation (Rideout, 2006). Our measurements of ovary thickness

395    and previtellogenic oocytes comparing spent and immature fish corroborate earlier observations

396    (Burton et al., 1997) in cod and we would commend this method to studies on cod maturity in wild

397    populations.

398

399 **Acknowledgements**

400

408

409 **References**

410

411 Armstrong, M.J.P., Conolly, P., Nash, R. D. M., Pawson, M. G., Alesworth, E., Coulahan, P. J.,

412     Dickey-Collas, M., Milligan, S. P., O'Neill, M. F., Witthames, P. R., Woolner, L., 2001. An

413     application of the annual egg production method to estimate the spawning biomass of cod

414     (*Gadus morhua* L.), plaice (*Pleuronectes platessa* L.) and sole (*Solea solea* L.) in the Irish

415     Sea. ICES J. Mar. Sci. 58, 183-203.

416 Beverton, R.J.H., Holt, S. J., 1957. On the dynamics of exploited fish populations. Fish. Invest. Ser.

417     II 19, 1-553.

418 Burton, M.P.M., Penney, R. M., Biddiscombe, S., 1997. Time course of gametogenesis in

419     Northwest Atlantic cod (*Gadus morhua*). Can. J. Fish. Aqua. Sci. 54, 122-131.

420 Emerson, L.S., Walker, M. G., Witthames, P. R., 1990. A stereological method for estimating fish

421     fecundity. J. Fish Biol. 36, 721-730.

Fitzhugh, G.R., Hettler, W. F., 1995. Temperature influence on postovulatory follicle degeneration in Atlantic menhaden, *Brevoortia tyrannus*. Fish. Bull. 93, 568-572.

Ganias, K., Nunes, C., Stratoudakis, Y. 2007. Degeneration of postovulatory follicles in the Iberian sardine (*Sardinia pilchardus*): structural changes and factors affecting resorption. Fish. Bull. 105,131-139.

Ganias, K., Nunes, C., Stratoudakis, Y. 2008. Use of late ovarian atresia in describing spawning history of sardine, *Sardina pilchardus*. J. Sea Res. 60, 244-249.

Godø, O.R., Michalsen K., 2000, Migratory behaviour of north east Arctic cod studied by use of data storage tags. Fish. Res. 48, 127-140.

Horwood, J.W., 1993. The Bristol Channel sole (*Solea solea* (L.)): A fisheries case study. Adv. Mar. Biol. 29, 215-368.

Hunter, J.R., Goldberg, S. R., 1980. Spawning incidence and batch fecundity in northern anchovy, *Engraulis mordax*. Fish. Bull. 77, 641-652.

Hunter, J.R., Macewicz, B. J., 1985a. Rates of atresia in the ovary of captive and wild northern anchovy, *Engraulis mordax*. Fish. Bull. 83, 119-136.

Hunter, J.R., Macewicz, B. J. 1985b. Measurement of spawning frequency in multiple spawning fishes. NOAA Tech.Rep.No, NMFS36, 79-94.

Hunter, J.R., Macewicz, B. J., 2003. Improving the accuracy and precision of reproductive information used in fisheries. In Report of the Working Group on Modern Approaches to Assess Fecundity and Maturity of Warm- and Cold-Water Fish and Squids. (O. S. Kjesbu, J. R. Hunter, and P. R. Witthames, eds.). Fisken og Hav. 12:57-68. The Institute of Marine Research, Bergen, Norway.

444 Kennedy, J., Witthames, P. R., Nash, R. D. M., 2007. The concept of fecundity regulation in plaice

445       (*Pleuronectes platessa* L.) tested on three Irish Sea spawning populations. Can. J. Fish.

446       Aqua. Sci.. 64, 587-601.

447 Kjesbu, O.S., 1989. The spawning activity of cod, *Gadus morhua* L. J. Fish Biol. 34, 195-206.

448 Kjesbu, O.S., Klungsøyr, J., Kryvi, H., Witthames, P. R., Greer, Greer Walker, M., 1991.

449       Fecundity, atresia, and egg size of captive Atlantic cod (*Gadus morhua*) in relation to

450       proximate body composition. Can. J. Fish. Aquat. Sci. 48, 2333-2343.

451 Kraus, G., Tomkiewicz, J., Deickmann, R. A., Köster, F., 2008. Seasonal prevalence and intensity

452       of follicular atresia in Baltic cod *Gadus morhua callarias* L. J. Fish. Biol. 72, 831-847.

453 Kurita, Y., Kjesbu, O. S., Meier, S., 2003. Oocyte growth and fecundity regulation by atresia of

454       Atlantic herring (*Clupea harengus*) in relation to body condition throughout the maturation

455       cycle. J. Sea Res. 49, 203-219.

456 Lockwood, S.J., Nichols, J. H., Dawson, W. A., 1981. The estimation of a mackerel (*Scomber*

457       *scomber* L.) spawning stock size by plankton survey. J. Plankton Res. 3, 217-233.

458 Mattson, N.S., Riple, T. H., 1989. Metomidate, a better anesthetic for cod (*Gadus morhua*) in

459       comparison with benzocaine, MS-222, chlorobutanol, and phenoxyethanol. Aquaculture 83,

460       89-94.

461 McEvoy, L.A., 1985. Double ovulatory cycles in some captive turbot, *Scophthalmus maximus* L. J.

462       Fish Biol. 26, 63-66.

463 McDonough, C. J., Roumillat, W.A, Wenner, C.A., 2005. Sexual differentiation and gonad

464       development in striped mullet (*Mugil cephalus* L.) from South Carolina estuaries. Fish. Bull.

465       103:601–619.

466  Morrison, C.M. 1990. Histology atlas of the Atlantic cod *Gadus morhua*: An Atlas. Part three.

467      Reproductive tract. Can. J. Fish. Aqua. Sci. Spec. Publ..

468  Murawski, S.A., Rago, P. J., Trippel, E. A., 2001. Impacts of demographic variation in spawning

469      characteristics on reference points for fishery management. ICES J. Mar. Sci. 58, 1002-

470      1014.

471  Murua, H., Kraus, G., Saborido-Rey, F., Witthames, P. R., Thorsen, A., Junquera, S. 2003.

472      Procedure to estimate fecundity of marine fish species in relation to their reproductive

473      strategy. J. NW Atl. Fish. Sci. 33, 33-54.

474  Neat F.C. Righton D.A., 2007. Warm water occupancy by North Sea cod. Proc. Roy. Soc. B 274,

475      789-798.

476  Óskarsson, G.J., Kjesbu, O. S., Slotte, A., 2002. Predictions of realised fecundity and spawning

477      time in Norwegian spring-spawning herring (*Clupea harengus*). J. Sea Res. 48, 59-79.

478  Palsson O., Thorsteinsson, V., 2001. Migration patterns, ambient temperature, and growth of

479      Icelandic cod (*Gadus morhua*): evidence from storage tag data. Can. J. Fish. Aqua. Sci 60,

480      1409-1423.

481  Rideout, R.M., 2006. Suppression of reproduction in Atlantic cod. Mar. Ecol. Prog. Ser. 320, 267-

482      277.

483  Rideout, R.M., Rose, G. A., Burton, M. P. M., 2005. Skipped spawning in female iteroparous

484      fishes. Fish Fish. 6, 50-72.

485  Saborido-Rey, F., Junqera, S., 1998. Histological assessment of variations in sexual maturity of cod

486      (*Gadus morhua* L.) at the Flemish Cap (north-west Atlantic). ICES J. Mar. Sci. 55, 515-521.

487    Schmidt-Nielsen, K., 1978. Animal Physiology. Adaptation and Environment. Cambridge

488        University Press.

489    Thorsen, A., Kjesbu, O. S., 2001., A rapid method for the estimation of oocyte size and potential

490        fecundity in Atlantic cod using computer-aided particle analysis system. J. Sea Res. 46, 295-

491        308.

492    Thorsen, A., Marshall, C. T., Kjesbu, O. S., 2006. Comparison of various potential fecundity

493        models for north-east Arctic cod *Gadus morhua*, L. using oocyte diameter as a standardizing

494        factor. J. Fish Biol. 69, 1709-1730.

495    Tomkiewicz, J., Tybjerg, L., Jespersen, A., 2003. Micro- and macroscopic characteristics to stage

496        gonadal maturation of female Baltic cod. J.  Fish Biol. 62, 253-275.

497    Tyler, C.R., Sumpter, J. P., 1996. Oocyte growth and development in teleosts. Rev. Fish Biol. Fish.

498        6, 287-318.

499    Witthames, P.R., Greer Walker, M., 1995. Determination of fecundity and oocyte atresia in sole

500        (*Solea solea*) (Pisces) from the Channel, the North Sea and the Irish Sea. Aqua. Liv. Res. 8,

501        91-109.

502    Witthames, P. R., Marshall., C.T. 2008. The importance of reproductive dynamics in fish stock

503        assessments. In: A. Payne, J. Cotter, and T. Potter (Eds.), Advances in Fisheries Science. 50

504        years on from Beverton and Holt. Blackwell Oxford pp. 306-324.

505    Witthames, P.R., Thorsen, A., Murua, H., Saborido-Rey, F., Greenwood, L., Dominguez, R.,

506        Korta,M., Kjesbu, O.S., 2009. Advances in fecundity methodology applied to some marine

507        fish. Fish. Bull. 107:48-6.

508

1 Table 1

2 List of experiments / aims, number of tanks, (NT), tank description (TD), number of female Atlantic cod per tank (NF), and starting values for mean

3 length [Lt (cm)], Fulton's condition factor [K(total weight / length 3 x 100)], gonadsomatic index [GSI] (ovary weight / total weight)], duration ($E_D$ days),

4 temperature regime (T $^{o}$C) controlled during the study (NM not measured). Mat 1-3 and Imm refer to single fish and BS and NS refer to Barents and North

5 Sea tanks respectively. All the fish were 2 years old and reared from captive brood stock at the Parisvannet aquaculture facility.

| Experiment / aim | NT | TD | NF | LT (SD) | K (SD) | GSI (SD) | $E_D$ | T (SD) |
|---|---|---|---|---|---|---|---|---|
| 1 Alpha atretic follicle | 2 | Concrete 15 m$^3$ in capacity 3 x | 25 | 50.5 (3.6) | 1.06 (0.10) | NM | 21 | 4.5 (0.3) |
| production and fate. | | 3 x 1.65 m deep. | 25 | 51.4 (3.5) | 1.09 (0.10) | NM | 21 | 8.1 (0.3) |
| 2 Postovulatory follicles | 3 | 200m$^3$ annual tank partitioned | Mat 1[1] | 38 | 1.15 | NM | 59 | 9.1 (0.2) |
| production and a comparison of | | into radial segments of 10m$^3$. | Mat 2[1] | 39 | 1.24 | NM | 74 | |
| spent and immature ovaries. | | | Mat 3[1] | 40.5 | 1.13 | NM | 99 | |
| | | | Imm[1] | 40.5 | 1.02 | NM | 74 | |
| 3 Fate of postovulatory and | 2 | BS 5m round x 1m deep | 20[2] | | | | 104 | 7.5-11.2 |
| residual vitellogenic follicles after spawning. | | NS 5m round x 1m deep | 20[2] | 50.3 (3.1)[3] | 0.087 (0.06)[3] | 0.025 (0.014)[3] | 104 | 9.9-16.4 |

6 [1] A male and female (Mat 1-3) spawning pair per tank segment. The immature female was held with surplus males in a 15 m$^3$ tank 3 x 3 x 1.65 m deep

7 [2] Five fish were taken for the first sample before the group was divided between the two tanks at the start of the experiment.

8 [3] Mean length, condition and GSI were calculated from a sample taken from the group before dividing between BS and NS

Table 2

Experiment 2: Duration of blastomere stages in Atlantic cod (*Gadus morhua*) based on

extrapolation to 9.1$^{o}$C from hours at 5 (h_5.5 $^{o}$ C) and 8.5 $^{o}$C (h_8 $^{o}$ C) and calculated rates

R_5.5 and R_8 respectively using a $Q_{10}$ temperature coefficient.

14

| Stage | No. of blastomers | h_8$^{o}$ C hours | h_5.5$^{o}$ C hours | R_8 | R_5,5 | Q10 | R9.1 $^{o}$ C | h9.1 $^{o}$ C hours |
|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 2 | 4 | 0.500 | 0.250 | 16.00 | 0.678 | 1.5 |
| 2 | 2 | 4 | 6 | 0.250 | 0.167 | 5.02 | 0.299 | 3.3 |
| 3 | 4 | 6 | 8 | 0.167 | 0.125 | 3.19 | 0.190 | 5.3 |
| 4 | 8 | 8 | 10 | 0.125 | 0.100 | 2.44 | 0.138 | 7.2 |
| 5 | 16 | 10 | 12 | 0.100 | 0.083 | 2.11 | 0.109 | 9.2 |
| 6 | 32 | 12 | 14 | 0.083 | 0.071 | 1.87 | 0.089 | 11.2 |
| 7 | 64 | 15 | 18 | 0.067 | 0.056 | 2.05 | 0.073 | 13.7 |
| 8 | 128 | 20 | 24 | 0.050 | 0.042 | 2.01 | 0.054 | 18.5 |

15

16

17

18    Table 3

19    Details of the date and fishing positions where wild Atlantic cod (*Gadus morhua*) were caught

20    using a bottom trawl in the North (NS) and Barents Seas (BS) and the results of the

21    histological analysis to determine the presence of post ovulatory follicles (POFs), residual

22    atretic vitellogenic follicles (cysts) and thickness of the tunica for maturity assessment. Cyst

23    and tunica data was not available (NA) in the Barents Sea collection.

| Date caught | Sea area | Latitude N$^o$ | Longtitude E$^o$ | Fish length (cm) | POF present | Cysts present | Tunica thickness (mm) | Mature / immature assessment |
|---|---|---|---|---|---|---|---|---|
| 26-Aug-06 | NS | 58.51 | 3.58 | 40 | N | N | 0.110 | immature |
| 26-Aug-06 | NS | 58.51 | 3.58 | 49 | N | N | 0.117 | immature |
| 01-Sep-06 | NS | 59.45 | 0.48 | 44 | N | N | 0.078 | immature |
| 01-Sep-06 | NS | 59.70 | 0.88 | 54 | N | N | 0.144 | immature |
| 01-Sep-06 | NS | 59.70 | 0.88 | 49 | N | N | 0.120 | immature |
| 22-Aug-06 | NS | 54.96 | 0.24 | 51 | Y | Y | 0.424 | Mature |
| 24-Aug-06 | NS | 60.36 | 5.21 | 58 | Y | N | 0.340 | Mature |
| 26-Aug-06 | NS | 58.51 | 3.58 | 50 | Y | Y | 0.676 | Mature |
| 01-Sep-06 | NS | 59.70 | 0.88 | 49 | Y | Y | 0.396 | Mature |
| 02-Sep-06 | NS | 61.00 | 1.22 | 80 | Y | Y | 0.882 | Mature |
| 16-Feb-06 | BS | 70.46 | 37.26 | 88 | Y | NA | NA | Mature |
| 16-Feb-06 | BS | 70.27 | 37.45 | 88 | Y | NA | NA | Mature |
| 17-Feb-06 | BS | 70.76 | 40.57 | 70 | Y | NA | NA | Mature |
| 18-Feb-06 | BS | 69.71 | 41.95 | 68 | Y | NA | NA | Mature |

24

25    Table  4

26    Experiment 3: ANOVA results after fitting  $y = a * \exp(-b * day)$  where y = POF area at

27    $day_t$ and a and b are area specific coefficients referring to the Barents Sea and North Sea

28    respectively with standard errors (SE), t values, P values and residual error.

29

| Parameter | Estimate | SE | t | P |
|---|---|---|---|---|
| a ($_{Barents\ Sea}$) | $1.42 \times 10^{-2}$ | $1.49 \times 10^{-3}$ | 9.566 | <0.001 |
| a ($_{North\ Sea}$) | $1.52 \times 10^{-2}$ | $1.76 \times 10^{-3}$ | 8.623 | <0.001 |
| b ($_{Barents\ Sea}$) | $2.58 \times 10^{-3}$ | $1.90 \times 10^{-3}$ | 1.363 | 0.182 |
| B ($_{North\ Sea}$) | $7.66 \times 10^{-3}$ | $2.58 \times 10^{-3}$ | 2.964 | <0.01 |
| Residual standard error  $3.44 \times 10^{-3}$ on 35 degrees of freedom | | | | |

30
31

32  Table 5

33

34  Details of atretic durations (±2 standard errors where available) and environmental

35  temperature recorded by this and previous studies.

36

| Species | Temperature C° | Experimental conditions | Estimated alpha atretic duration (days) | Authors |
|---|---|---|---|---|
| *Engraulis mordax* | 16 | Starvation and group observation | 8 | Hunter and Macewicz (1985) |
| *Gadus morhua* | 9 | Natural spawning | 10 | Kjesbu et al. (1991) |
| *Clupea harengus* | 4.2 – 11 | Wild population | July-October 5.8 | Kurita et al. (2003) [1] |
| | 6.8-10 | | October-November 8.7 | |
| | 5.8-7.2 | | November-January 7.8 | |
| | 5.8-6.7 | | January-February 9.1 | |
| *Gadus morhua* | 4.5 | Lab individual observation | 5.3 ± 2.5 | This publication |
| | 8.1 | | 9.7 ± 4.9 | |
| | 4.5-8.1 | | 7.5 ± 2.9 | |

37

38  [1] Used results based on atresia intensity raised by a Disector correction of 1.27

**Figure legends**

Fig. 1

41 Scatter plots showing the proportion of alpha (α) and beta + gamma (β+ γ) to normal

42 vitellogenic follicles (filled and open circles respectively) found in biopsy samples taken from

43 Atlantic cod (*Gadus morhua*) in Experiment 1 kept in water controlled to 4.5 [standard

44 deviation (SD) 0.3] in the upper two rows and 8.1 (SD 0.3) $^{o}$C (lower 2 rows). The dashed and

45 dotted horizontal lines show the starting (reference) level of αF and βF+ γF when the first

46 biopsy was taken on the 5 (4.5$^{o}$C water) and 4 (8.1$^{o}$C water) of March. Upward and

47 downward arrows indicate when the proportion of αF and βF+ γF exceeded the reference

48 levels in each case to determine the duration (days) of the αF stage shown at the top of each

49 panel and as a grey band between the arrows.

50 Fig. 2

51 Sections of ovary biopsy taken from captive Atlantic cod (*Gadus morhua*) in Experiment 1

52 stained with periodic acid Schiff's and Mallory trichrome illustrating stages of follicle

53 regression. Alpha atresia (α) in early and late vitellogenic follicles (upper left and right

54 panels) is indicated by small breaks in the chorion (CB arrow) which continues to fragment

55 (FC block arrows) and disappears by the beta + gamma atresia stages (βF+ γF). Yolk granules

56 (YG arrow) also persist through the α stage but are absent in βF+γF. POF (bottom panels)

57 have a convoluted outline and also a clearly defined unstained central area (the lumen)

58 surrounded by a PAS staining basement membrane that becomes more pronounced as the

59 POF ages from early (EP) to later stages (LP). The PAS membrane (arrow) was still visible

60 but indistinct in βF+γF (bottom right panel. The scale bar = 1000μm.

61

62 Fig 3

63 Sections of ovary biopsy (upper left panel) or whole ovary (upper right and middle panels)

64 taken from captive Atlantic cod (*Gadus morhua*) in Experiment 2 (Table 1) stained with

65 periodic acid Schiff's and Mallory trichrome) illustrating the range of post-ovulatory follicle

66  (POF) morphology. POF taken by ovary biopsy (female Mat 1) less than 12 hours post

67  spawning (upper left) have a large lumen bordered by the follicle comprising granulosa (G)

68  and thecal (T) layers separated by the PAS stained basement membrane (arrow). Early and

69  late stage POF accumulate (top right panel) throughout spawning (ovary section Mat 2). The

70  ovary tunica (T) is clearly much thinner in ovary section from female Imm (left middle panel)

71  compared to female Mat 3 (right middle panel) respectively that also contain POF aged 45 or

72  more days post spawning (arrows).  The lower two panels show examples of encapsulated

73  follicle cysts (EC) comprising the residual chorion (C), yolk granules (YG arrow) and POF >

74  150 days old (arrows) from ovaries in wild mature post spawning fish (Table 3). The scale bar

75  = 1000μm.

76  Fig 4

77  Upper three panels: Cumulative production of spawned eggs from Atlantic cod (*Gadus*

78  *morhua*) Mat 1 -3 in Experiment 2.  The dates when biopsy samples were removed to study

79  post ovulatory follicle (POF) production and when the fish were killed are indicated by

80  vertical lines along the time axis.  Lower three panels: Numbers of residual follicles classified

81  as normal (black bars) hydrated (grey bars) and atretic (white bars) found in the ovaries of

82  Mat 1 – 3.

83

84  Fig. 5

85  Temperature regime maintained during Experiment 3 lasting from 30 April until 12 August.

86  The black and open circles refer to the North and Barents Sea simulations respectively whilst

87  the triangles on the base line show when Atlantic cod (*Gadus morhua*) were sampled on Day

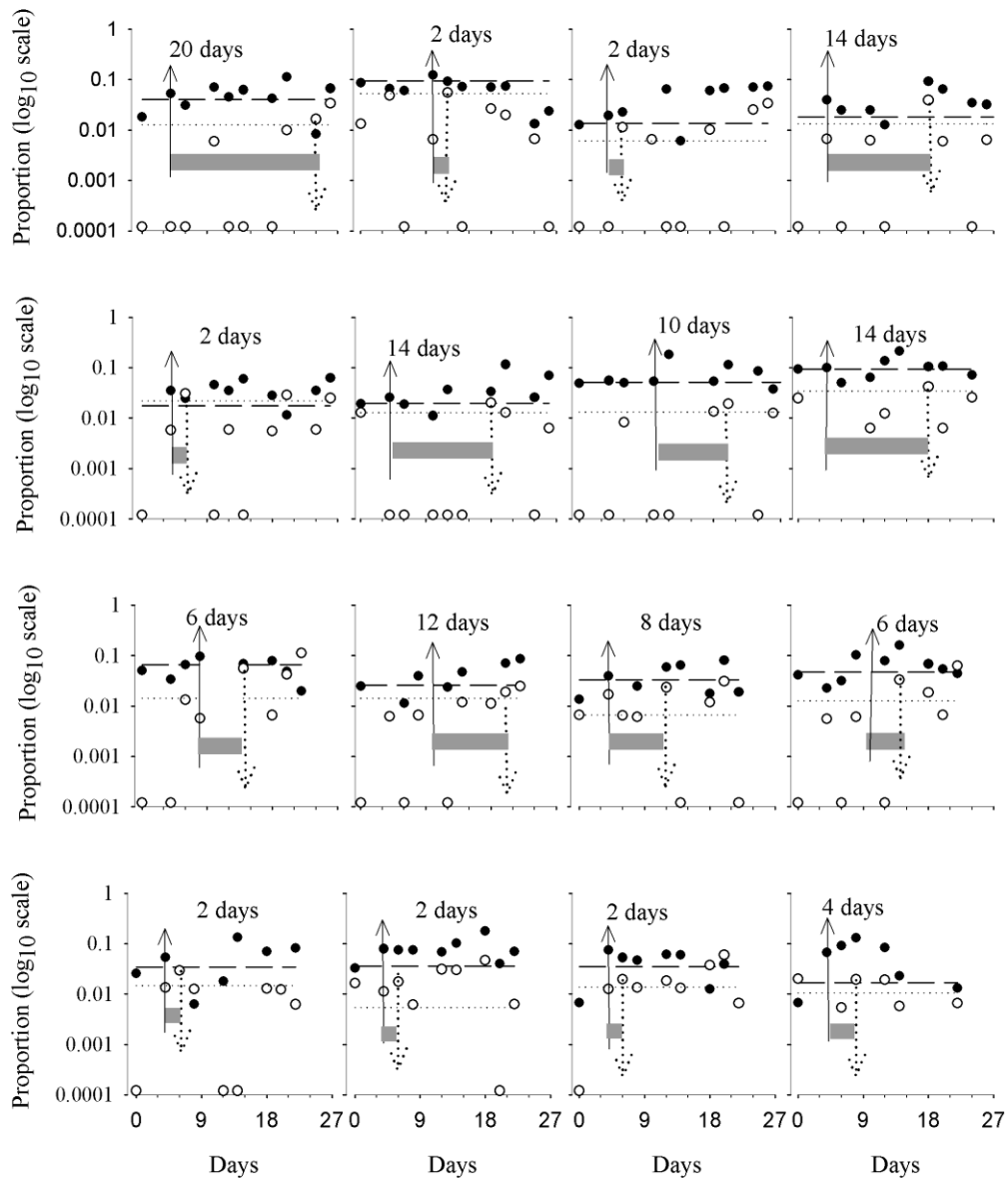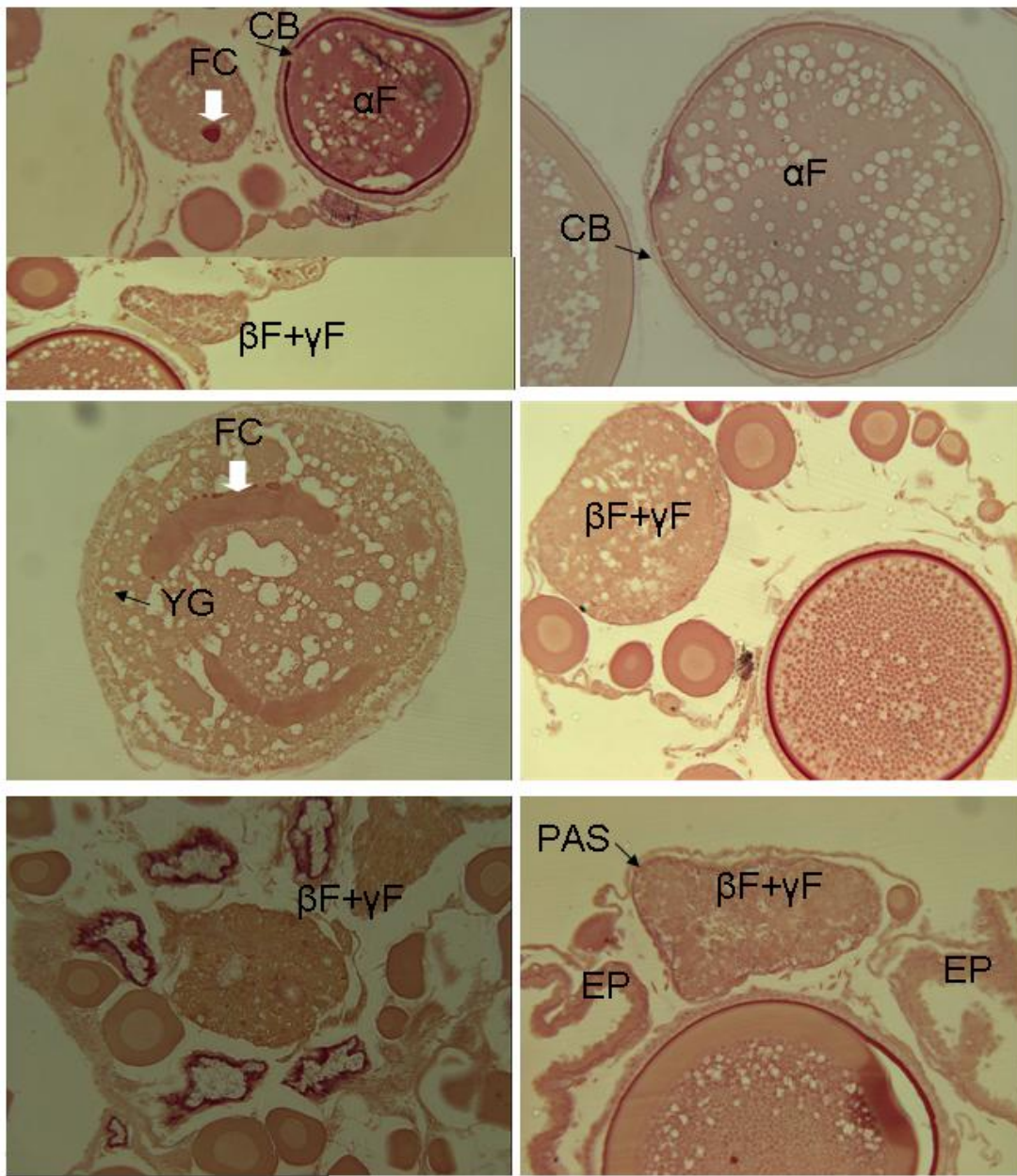88  0, 14, 28, 56 and 104, respectively.

89

90  Fig. 6

91   Experiment 3: Area of leading post ovulatory follicle (POF) cohort measured in histological

92  sections prepared from ovaries of Atlantic cod (*Gadus morhua*) kept in 5 m tanks simulating

8

93 (A) North Sea and (B) Barents Sea spring to early autumn warming regime sampled at

94 internals from 30 April to 12 August. The solid lines where fitted with an exponential decay

95 model y = a * exp (-b * day) using area specific coefficients shown in Table 4 and the dotted

96 lines show ± 95% confidence limits. The filled data points apply to experimental fish kept in

97 the North Sea (upper panel) and the Barents Sea (lower panel) and the open circle data points

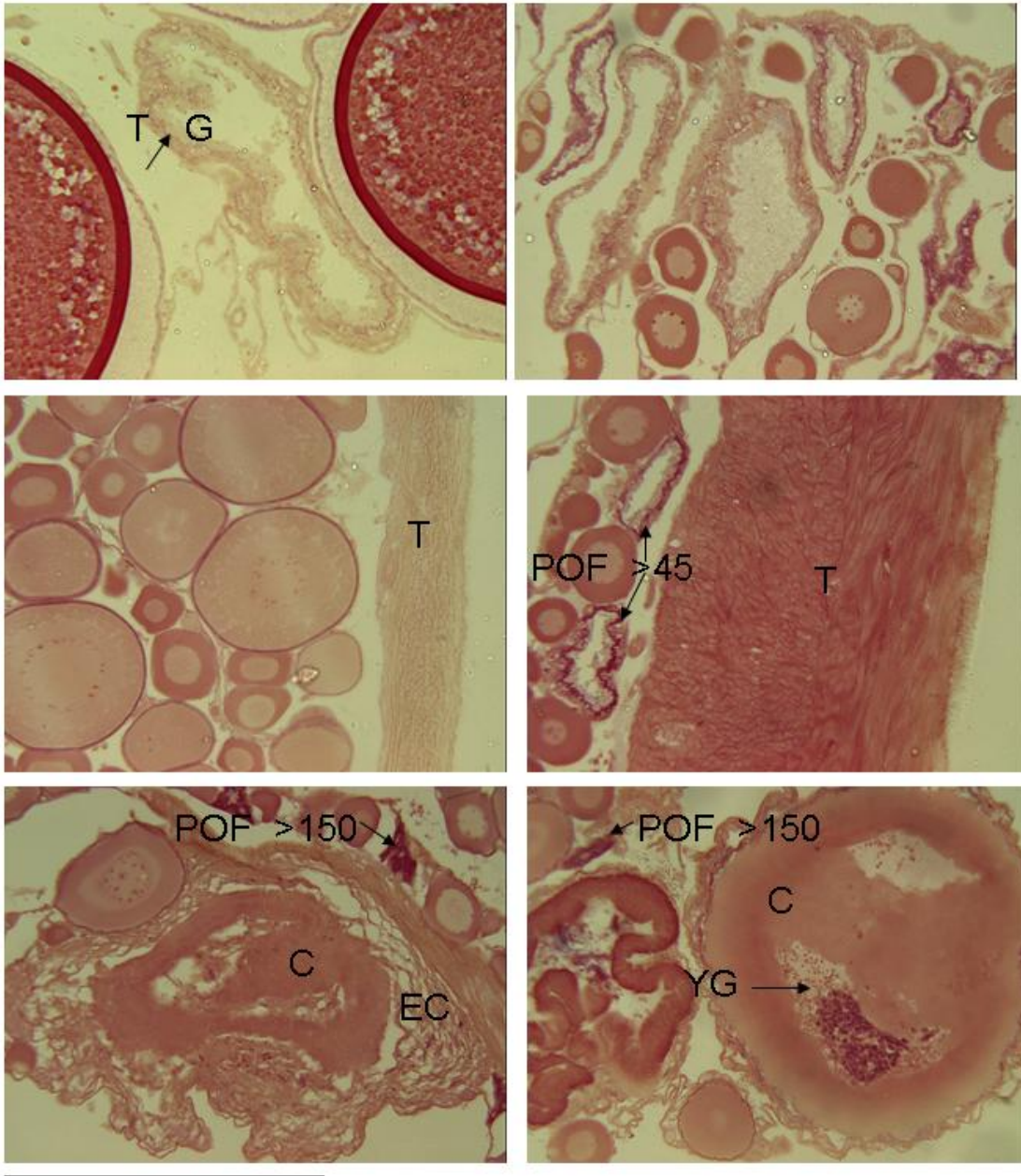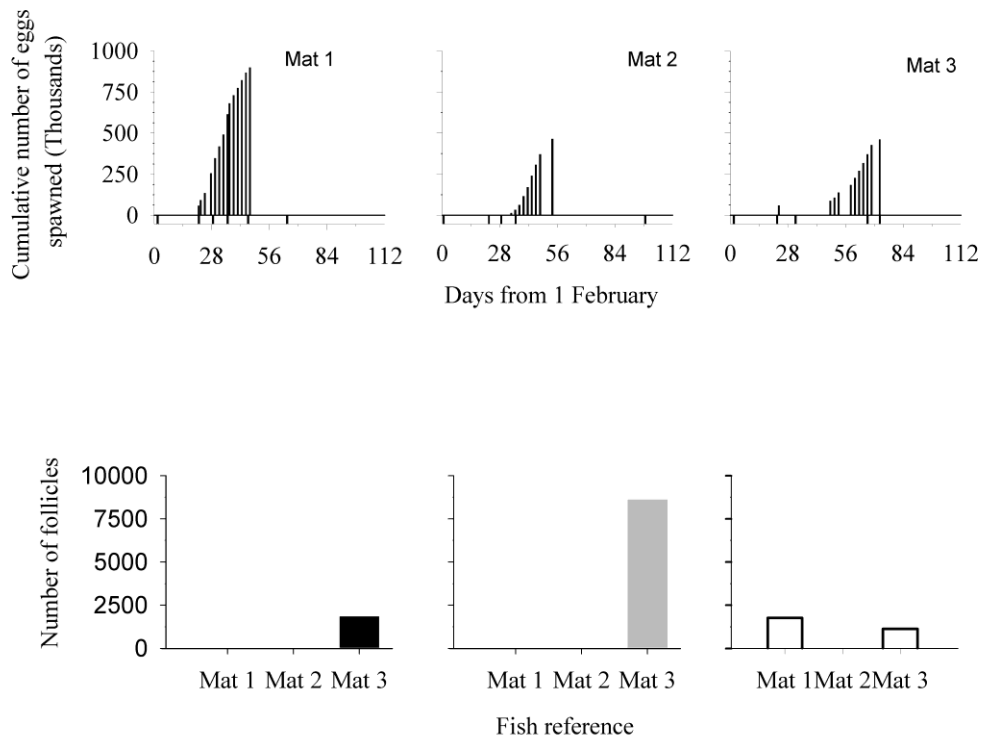98 apply to wild fish (Table 3) collected in each area.

99

100

103    Fig. 2

108    Fig. 3

109

110

111    Fig. 4



112

113

114    Fig. 5



115

116    Fig. 6

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