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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) °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).

In Experiment 2 we took biopsy samples at intervals and monitored egg production from individual females accompanied by a male and used the stage of egg development to age POFs found in the biopsy samples. The females, some immature, were killed at intervals, up to 45 days post spawning, and then the biopsy and ovary samples were stained by periodic acid Schiffs' reagent to prepare descriptions of POFs aged from 11 hours to 45 days old. Spent female ovaries contained POFs, and a thicker ovarian wall (tunica) exceeding 0.34 mm whilst immature fish lacked POFs and their ovary tunica was thinner (less than 0.15mm). In Experiment 3 the persistence of POFs was monitored in a simulated North Sea (10-16.1 $^{\circ}$C) and Barents Sea (7.5-11.2°C) regime using ovary sections stained by periodic acid Schiffs' reagent. In both regimes the POFs regressed at a temperature sensitive rate during the experiment lasting 104 days. Some αF from large VF persisted longer than expected (more than four months after spawning) and were called cysts based on their appearance and greater expected lifetime. These histological characteristics were successfully applied to assess maturity of wild cod caught on surveys in the North and Barents Seas after an assumed 150 and 310 days respectively after the spawning season. Taken together this article presents reliable figures on the lifetime of atretic and post-ovulatory follicles as well as variation in ovarian thickness with spawning experience, which will be most useful input in the further work to assess reproductive potential.

1. Introduction

Female reproductive potential plays a pivotal role in the capacity of wild fish populations to sustain their numbers when facing heavy fishing mortality so it is important to establish the dynamics of egg production. Although Virtual Population Analysis (VPA) makes it possible to assess numbers by age class (Beverton and Holt, 1957) it is also important that we assess the relationship between stock and reproductive potential (Murawski et al., 2001;Witthames and 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 $$B_s = \frac{TEP}{Fr} \quad (1)$$

80 where TEP = population total egg production and

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

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

86 where D is the number of days alpha atretic follicles take to regress to the beta stage, αFpop the
87 population average of the proportion of yolk follicles in the alpha atretic stage (αF), Sp (days)
88 spawning duration (Kjesbu et al., 1991; Horwood, 1993), and P is the proportion of females in the
89 population containing αF. 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 αF 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

term follicle referring to both the oocyte and outer follicle layers (Tyler and Sumpter, 1996). In Experiment 1 we assessed atretic vitellogenic follicle production by studying changes in the ratio of normal to alpha, and a combined beta and gamma stage using published criteria (Hunter and Macewicz, 1985a). We exposed the fish to temperatures considered typical of those experienced by North Sea and Barents Sea cod stocks so that the results would be relevant to a range of habitat occupied by this species. Experiment 2 monitored egg production, and POF regression in mature fish. The ovaries from immature and mature females, known to have spawned, were compared in relation to ovary tunica thickness, residual αF, atretic follicles and POFs. In Experiment 3 the persistence of POFs was studied simulating a Barents Sea and North Sea spring warming cycle from the end of April to August. Consideration was then given to using the above spawning markers to identify spent mature and immature wild cod collected 6 (North Sea) and 11 months (Barents Sea) after the previous spawning season.

2. Materials and methods

2.1. Experiment 1: αF production and fate

Fish were sedated in 5 mg l^{-1} metomidate dissolved in oxygenated sea water (Mattson and Riple, 1989) during all the handling and measurement operations in the experiment (Table 1). Prior to starting the experiment a PIT tag (Destron Fearing, USA), was inserted subcutaneously into each fish for subsequent identification and a biopsy sample was removed using a Pipelle de Cornier® [Prodimed, Neuilly En Thelle, Picardie, France (Witthames et al., 2009)], from the ovary by catheterisation through the genital pore (McEvoy, 1985; Kjesbu, 1989). The total mass (g) and total length (cm) of each fish were also measured in this preparatory work. Each biopsy sample was fixed in 3.6 % formaldehyde solution buffered to pH 7.0 by 0.1 M sodium phosphate (NBF) for a minimum of two weeks before further processing. To identify and select only maturing fish for the 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^{o}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

Preparation of fish for the experiment (Table 1) followed the procedure detailed in Experiment 1. Prior to the start of the experiment the fish were fed on moderate rations (Kjesbu et al., 1991) and transferred to the experimental tanks when feeding was discontinued to monitor egg production (Kjesbu 1989).

At the start of the experiment a biopsy was taken following brief sedation, as in Experiment 1, and examined to determine sex and maturity status for selection of females used in the study (Table 1). Processing of biopsy samples followed the same protocol as Experiment 1. POFs were identified using criteria for multiple spawning fish (Hunter and Macewicz, 1985b) and specifically for cod (Murua et al., 2003) applied to PAS stained sections (Fig. 3).

Further biopsy samples were removed at intervals (Fig. 4) whilst egg production from each female was monitored so that we could link POF persistence and morphology with a known spawning history. Monitoring egg production involved estimating the number of eggs in each batch, F_r and the time of spawning based on temperature-specific egg development rates (Table 2) using published data and methods (Kjesbu, 1989).

The experiment was terminated (Table 1) to remove the ovaries which were fixed for a minimum of two weeks prior to cutting out whole cross sections 5 mm thick mid way from one end. Each cross section was processed as the biopsy samples, in order to estimate the residual VF, and αF by a stereometric method (Emerson et al., 1990). Measurements of the ovary tunica thickness, maximum previtellogenic oocyte diameter (repeated in seven microscopic fields) were made using Myrmica 4 software with a resolution of 3.5 µm per pixel in each case.

2.3 Experiment 3: fate of postovulatory and residual vitellogenic follicles

This experiment was started (Table 1) in spring by killing five females, using the same procedure as in Experiment 2, from a group that had just completed the annual

spawning cycle. This group was then divided between two tanks where the temperature was controlled to simulate a North Sea or Barents Sea spring to summer warming regime (Fig. 5). Fish were fed from the start of the experiment to satiation twice weekly until the experiment finished in late summer after 104 days. Further samples of five fish from both tanks (Fig. 5) were killed at intervals until the end of the experiment. Each ovary sample was processed as the biopsy samples in Experiment 2, to prepare stained histological slides to determine the rate of POF regression and to look for the presence of residual vitellogenic follicles (together referred to as spawning markers).

All POFs encountered whilst scanning across the section were measured using a polygon function (Myrmica 4 freeware [myrmica.co.uk]) to define the cross section area, until 20 observations were in the data set. The mean size of the largest two POFs from each sample was taken as the leading POF cohort and assumed to originate from the last ovulation. The rate of POF regression was investigated using an exponential decay model:

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

where y = POF area and we test whether the same or area specific coefficients are required for the Barents Sea and North Sea data to give the best fit.

2.4. Spent-recovering wild fish ovary histology

Cod were taken from trawl hauls made during the 'International bottom trawl survey' (IBTS) in the third quarter from the North Sea and during the 'winter survey' in the first quarter from the Barents Sea (Table 3). In each case the ovary was removed and a whole or part cross section was fixed in NBF. The fixed tissue was processed into stained slides as above. These slides were examined for the presence of POFs, residual atretic vitellogenic follicles (cysts) assumed to have originated from the last spawning which occurred approximately 150 and 305 days previously in the

Northern North Sea and off the Lofoten Isles respectively. The ovary tunica thickness, when present in the sample, and the cross section area of POFs was measured as in Experiment 2 and 3 respectively.

3. Results

3.1 Experiment 1: αF production and fate

The use of PAS Mallory to stain biopsies made it easy to visualise the transition of VF to αF based on the fragmentation of the chorion and dissolution of the yolk (Fig. 2). Although the PAS positive basement membrane was visible between the thecal and granulosa layers throughout regression of VF to βF + γ F, it never became enlarged or pronounced as in older POFs. Vacuoles and intercellular cavities were apparent in the βF + γ F stage but were spread out and small compared to the large unstained lumen making up the central part of the POF (Figs. 2 and 3).

Only 8 of the 25 fish in each temperature regime (Table 1) produced αF and then βF + γ F to exceed the αF and βF + γ F reference levels (Fig. 1). The αF stage was approximately twice as abundant compared to the βF + γ F stage in both regimes but there was also considerable variation in consecutive biopsy samples. There was an insignificant statistical effect (P=0.125) of temperature on the mean duration (days) of αF, for all fish in the group although it was longer in the cooler water 9.7 days [2 standard error (2 SE) 4.9] compared to 5.3 days (2 SE 2.5). The combined data from each temperature regime gave an αF duration of 7.5 days (2 SE 2.9).

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

The two females Mat 1 and Mat 2 produced mostly 100% fertile regular batches of eggs, spawning for the first time on the 22 February and 4 March respectively, whilst female Mat 3

produced a small batch on the 24 February before more regular batch production from 21 March (Fig. 4). Biopsy samples taken prior to spawning, mostly from Mat 1, contained no POF like structures but POFs appeared in all biopsies with increasing abundance following the start of spawning. The POFs found in the first biopsy from Mat 1, were aged between 10.2 to 12.45 hours old because the eggs at 32 blastomere stage originated from the first ovulation.

Thus our collection of biopsy samples and whole ovary sections were taken from 10 to 12.45 hours post spawning until 45 days after spawning had finished (Fig. 4). The POF aged at 10 to 12.45 hours old had collapsed to a thin curly band of granulosa and thecal cells lying each side of a PAS stained basal membrane around a large lumen typically 530 µm across its longest axis (Fig. 3). In Mat 3, killed just before spawning had finished (Fig. 4), there was a range of POF structures originating from the regular succession of egg batches produced during the experiment. The largest POF appeared similar to the example found in the first biopsy after spawning from Mat 2, but others showed a gradation of size. Because we found that POFs persisted for at least 45 days post spawning in the spent ovary of Mat 1 the range of POF structure in Mat 3 show the accumulation over all the preceding spawning events for this fish. The smallest POF still showed pronounced PAS staining of the residual basement membrane and a clearly defined central lumen.

Comparing ovaries from near spent or spent females (Mat 2 and 3) with immature females it was noted that larger previtellogenic follicles were present in the immature fish (up to 185 (2SE 6) and 224 (2SE 12) µm, respectively) compared to 131 (2SE 18) µm in the two spent fish (Fig. 3). The ovary tunica was much less developed, 120 µm thick, in the immature fish and up to 650 µm thick in the ovary of Mat 2. Also in Mat 2 large atretic vitellogenic follicles were aggregated into a mass in some cases so that it was difficult to see the boundary of each follicle.

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

The temperature regimes imposed in the tank water, simulating the Barents Sea and North Sea spring summer warming regime, differed by 2.6^{o}C at the start of the experiment (Table 1) and diverged to 4.9 ^{o}C, (based on a 10 day moving average) when the final sample was taken 104 days later (Fig. 5). POF shrinkage rates were significantly different in the two temperature regimes (Table 4) so that the distribution of POF areas (Fig. 6) became marginally significantly different after 104 days (t= 1.973, degrees of freedom = 5.56, P=0.0998 two sample Welch two sample student t test). In each case the lumen of the POF was evident throughout regression whilst the area of PAS staining was pronounced at first but became progressively reduced though still visible when the last sample was taken in August (Fig. 3). Surprisingly, atretic follicles, referred to as cysts, were still seen in some of the ovary sections taken in August from both temperature regimes. The follicles concerned showed a thickened chorion, and occasionally, some yolk granules. The outer follicle layers were fibrous with unstained void areas (Fig. 3).

3.4. Spent-recovering wild fish ovary histology

Extrapolating the separate temperature POF regression models (Table 4) to the number of days post spawning, assumed 150 and 305 days after sampling for the Barents Sea and North Sea respectively, suggested that POFs should still be visible. This was verified by a comparison of the predicted and observed POF area (Fig. 6) with the latter being above or within the 95% confidence interval of the prediction. Also seen in spent ovaries were large follicle cysts and thickened tunica (Fig. 3) that were very similar in appearance when compared to spent females in Experiment 2. Mostly the cysts were discrete objects in the cross section but in some cases cysts in close proximity were aggregated into a mass where it was not possible to discern boundaries. Based on the presence or absence of these spawning markers it was possible to distinguish between immature or post spawning ovaries (Table 3).

4. Discussion

When we planned Experiment 1 there was little information on the temperature experienced by free living Atlantic cod to decide on relevant experimental temperature regimes However, this information is now gradually building up with the use of data storage tags in different waters (Godø and Michalsen, 2000; Palsson and Thorsteinsson, 2003, Neat and Righton, 2007). These articles show that the temperature range used in Experiment 1 were typical or slightly above temperatures experienced by stocks, from the northern North Sea to north Iceland just prior to, or during spawning (David Righton Cefas, UK, personal communication.).

Our estimated atretic follicle (αF) duration would therefore be widely applicable although we were disappointed by the low precision around the mean duration (D). Although data from wild Atlantic cod populations show 1/3 of fish sampled contain αF (Armstrong et al., 2001; Kraus et al., 2008; Witthames et al., 2009) we expected a higher proportions given the stress of the repeated biopsy sampling. Higher levels of individual αF and older atretic stages (βF+γF) would be obtained by an unbiased but much more laborious Disector method (Kjesbu et al. this monograph) but would likely be of marginal interest in the present context. This approach was rejected because we were concerned with relative changes of αF and βF+γF compared to normal vitellogenic follicles (VF). Although we accept αF and βF+γF would be undersampled the error would be a constant bias rather than subject to change during the short period of the experiment.

The production of αF and βF + γ F, in relation to the reference value, was similar though slightly less in the case of βF + γ F suggesting the βF + γ F stage maybe shorter than that recorded for αF. Despite the effect of undersampling the βF + γ F stage part of the explanation may be because the most durable part of the follicle, the chorion, has disappeared by the end of the αF stage, so there is little solid material remaining to identify the final extinction phase of the follicle. However, an

alternative explanation has been reported in striped mullet [(*Mugil cephalus*) McDonough et al. 2005] and sardine [(*Sardinia pilchardus*) Ganias et al., 2007]: accumulation of βF+γF moves from the epithelium and concentrates medially in the ovarian lamellae and therefore may be under sampled by the biopsy pipelle.

The αF duration for each temperature regime determined from our experiments shows some consistency compared with earlier reports, given the range of temperatures, maturity stages and species (Table 5). It is likely that the rate of αF regression will follow the Q_{10} rule (Schmidt-Nielsen, 1978) so that its duration will be inversely proportional to water temperature but follicle size, depending on maturity stage, will confound the effect of temperature. For example αF duration in Atlantic herring varied between 5.8 days, during early ovary maturation of small follicles (500 µm) in July- October, to 9.1 days just prior to spawning when the follicles are approaching 1300 µm (Kurita et al., 2003. However, there is also inconsistency between the anchovy *Engraulis mordax* rate [8 days at 16^{o}C (Hunter and Macewicz 1985a)] where the developing follicles are smaller compared to cod reported from 7.5 (our data) to 10 days (Kjesbu et al., 1991) at 4.5-9 ºC.

The persistence of regressing αF that were still present 150 days post spawning in both experimental and wild fish was not expected based on all this evidence. We consider that these structures should be more accurately referred to as cysts (Tomkiewicz et al., 2003) as they are not following the normal dynamic of αF regression. Although our results confirm a recent study on sardine (Ganias et al., 2008) that αF was a short term stage we believe that the largest vitellogenic follicles, failing to enter final maturation, become encysted. In some cases we saw parts of cyst aggregations resembling the much later delta stage of atresia (Hunter and Macewicz 1985a), i.e., without clearly defined boundaries between each follicle.

For the first time we report on changes in POF morphology and size from 12 hours after the first spawning to 45 days post spawning in individual cod and over 104 days during the post spawning period by sampling groups of cod. Although the data from the Barents and North Sea did not

separate completely during the experiment (P=.1) the distributions were moving apart and would probably have separated if the experiment had lasted another 15 days. Our results support the classical work describing the ageing process of POFs in captive anchovy (Hunter and Goldberg, 1980) and more recent studies in sardine where POF perimeter and shape were shown to shrink rapidly (Ganias et al., 2007) but over a time scale measured in a few days. A 3D study on cod POF shape (Korta et al., this monograph) also makes an interesting comparison. However, our observation that POFs last months is quite different to the situation reported in anchovy (Hunter and Macewicz 1985b) or sardine (Ganias et al., 2007). POFs in anchovy were thought to become very reduced and difficult to distinguish from βF or γ F by the second day (Hunter and Macewicz 1985b). This may be more exaggerated if the ovary is fixed whole and subject to compression by the ovary tunica rather than in small fragments (Witthames et al., 2009; Korta et al., this monograph). In the case of cod we found the use of PAS stain and a central lumen that we followed throughout POF regression made distinction between POF and βF or γ F unambiguous. The central lumen was also considered an important criterion to distinguish POF from βF or γ F in the case of sardine (Ganias et al., 2007). We also noticed that old POFs were very numerous and of similar size and shape, whilst βF + γF were present in relatively low numbers and appeared with a less convoluted outline compared to POFs.

Temperature has previously been shown to effect POF regression (Fitzhugh and Hettler, 1995; Ganias et al., 2007) in warm-water species Atlantic menhaden (*Brevoortia tyrannus*) and sardine living at 14.8 to 20^{o}C. The presence of POFs has, however, also been used to indicate previous spawning events further away in time; in Flemish Cap cod POFs were stated to be present in the ovaries 3-4 months after spawning (Saborido-Rey and Junqera, 1998). Our results agree with these field results and provide a means to hindcast the time elapsed since spawning based on POF profile area measured in section.

Comparing the temperature regimes we imposed during Experiment 3 it is now apparent that both groups were exposed to warmer water than would be expected (Godø and Michalsen, 2000; Neat and Righton, 2007). The North Sea regime was probably a few degrees higher than normal during the post spawning season but in the summer more typical of the shallower Southern region than the Northern North Sea. The Barents Sea simulation was probably several degrees warmer than what would be expected when the fish move north into the Barents Sea after the spawning season.. However, based on the experimental data there should be no problem detecting POFs at least 150 days post spawning though in the more northerly cold areas this period could be extended, perhaps to over a year. The POFs found in wild fish caught in the Northern North Sea (above 57^{o} North) about 150 days post spawning, were mostly larger and outside the predicted confidence limits. Temperature data from cod fitted with storage tags caught in the Northern North Sea (Neat and Righton, 2007) show they live in colder water during the summer depending on locality [mean 7.6 (SD 1.86) -9.5 (SD 1.91) ^{o}C] reflecting more closely the Barents Sea simulation. The situation is further complicated because some cod frequent mostly shallower warmer water whilst others occupy deeper colder offshore water during the summer even though they spawn in similar temperature regimes [Icelandic cod: around 7^{o}C (Palsson and Thorsteinsson, 2003)].

We have now applied the PAS stain to detect the presence of POFs in several species both immediately post spawning and also after many months have elapsed (Skjæraasen et al., In press; Witthames unpublished data). In a closely related gadoid, Atlantic haddock (*Melanogrammus aeglefinus*), POFs were found in sections prepared from ovary samples taken in the third quarter IBTS survey in the North Sea several months after their assumed last spawning season. In contrast Scombroids such as Atlantic mackerel (*Scomber scombrus*) or Carangidae such as horse mackerel (*Trachurus trachurus*) or Clupeids such as Atlantic herring produce POFs that do not stain as effectively with PAS and appear to disappear within days, being absent in spent or partially spent 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

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References

Armstrong, M.J.P., Conolly, P., Nash, R. D. M., Pawson, M. G., Alesworth, E., Coulahan, P. J., Dickey-Collas, M., Milligan, S. P., O'Neill, M. F., Witthames, P. R., Woolner, L., 2001. An application of the annual egg production method to estimate the spawning biomass of cod (*Gadus morhua* L.), plaice (*Pleuronectes platessa* L.) and sole (*Solea solea* L.) in the Irish Sea. ICES J. Mar. Sci. 58, 183-203.

Beverton, R.J.H., Holt, S. J., 1957. On the dynamics of exploited fish populations. Fish. Invest. Ser. II 19, 1-553.

Burton, M.P.M., Penney, R. M., Biddiscombe, S., 1997. Time course of gametogenesis in Northwest Atlantic cod (*Gadus morhua*). Can. J. Fish. Aqua. Sci. 54, 122-131.

Emerson, L.S., Walker, M. G., Witthames, P. R., 1990. A stereological method for estimating fish fecundity. J. Fish Biol. 36, 721-730.

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

424 Ganias, K., Nunes, C., Stratoudakis, Y. 2007. Degeneration of postovulatory follicles in the Iberian
425 sardine (*Sardinia pilchardus*): structural changes and factors affecting resorption. Fish. Bull.
426 105,131-139.

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

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

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

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

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

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

439 Hunter, J.R., Macewicz, B. J., 2003. Improving the accuracy and precision of reproductive
440 information used in fisheries. In Report of the Working Group on Modern Approaches to
441 Assess Fecundity and Maturity of Warm- and Cold-Water Fish and Squids. (O. S. Kjesbu, J.
442 R. Hunter, and P. R. Witthames, eds.). Fisken og Hav. 12:57-68. The Institute of Marine
443 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

Table 1

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

[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

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

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

9

Table 2

Experiment 2: Duration of blastomere stages in Atlantic cod (*Gadus morhua*) based on extrapolation to 9.1°C from hours at 5 (h_5.5 °C) and 8.5 °C (h_8 °C) and calculated rates R_5.5 and R_8 respectively using a Q_{10} temperature coefficient.

Stage	No. of blastomers	h_8° C hours	h_5.5° C hours	R_8	R_5,5	Q10	R9.1 °C	h9.1 °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

Table 3

Details of the date and fishing positions where wild Atlantic cod (*Gadus morhua*) were caught using a bottom trawl in the North (NS) and Barents Seas (BS) and the results of the histological analysis to determine the presence of post ovulatory follicles (POFs), residual atretic vitellogenic follicles (cysts) and thickness of the tunica for maturity assessment. Cyst 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

Table 4

Experiment 3: ANOVA results after fitting $y = a * \exp(-b * day)$ where y = POF area at day_t and a and b are area specific coefficients referring to the Barents Sea and North Sea respectively with standard errors (SE), t values, P values and residual error.

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

Table 5

Details of atretic durations (±2 standard errors where available) and environmental temperature recorded by this and previous studies.

Species	Temperature C^o	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)
	6.8-10		October-November 8.7 [1]	
	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	

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

Figure legends

Fig. 1

Scatter plots showing the proportion of alpha (α) and beta + gamma (β+ γ) to normal vitellogenic follicles (filled and open circles respectively) found in biopsy samples taken from Atlantic cod (*Gadus morhua*) in Experiment 1 kept in water controlled to 4.5 [standard deviation (SD) 0.3] in the upper two rows and 8.1 (SD 0.3) ^{o}C (lower 2 rows). The dashed and dotted horizontal lines show the starting (reference) level of αF and βF+ γF when the first biopsy was taken on the 5 (4.5^{o}C water) and 4 (8.1^{o}C water) of March. Upward and downward arrows indicate when the proportion of αF and βF+ γF exceeded the reference levels in each case to determine the duration (days) of the αF stage shown at the top of each panel and as a grey band between the arrows.

Fig. 2

Sections of ovary biopsy taken from captive Atlantic cod (*Gadus morhua*) in Experiment 1 stained with periodic acid Schiff's and Mallory trichrome illustrating stages of follicle regression. Alpha atresia (α) in early and late vitellogenic follicles (upper left and right panels) is indicated by small breaks in the chorion (CB arrow) which continues to fragment (FC block arrows) and disappears by the beta + gamma atresia stages (βF+ γF). Yolk granules (YG arrow) also persist through the α stage but are absent in βF+γF. POF (bottom panels) have a convoluted outline and also a clearly defined unstained central area (the lumen) surrounded by a PAS staining basement membrane that becomes more pronounced as the POF ages from early (EP) to later stages (LP). The PAS membrane (arrow) was still visible but indistinct in βF+γF (bottom right panel. The scale bar = 1000μm.

Fig 3

Sections of ovary biopsy (upper left panel) or whole ovary (upper right and middle panels) taken from captive Atlantic cod (*Gadus morhua*) in Experiment 2 (Table 1) stained with periodic acid Schiff's and Mallory trichrome) illustrating the range of post-ovulatory follicle

(POF) morphology. POF taken by ovary biopsy (female Mat 1) less than 12 hours post spawning (upper left) have a large lumen bordered by the follicle comprising granulosa (G) and thecal (T) layers separated by the PAS stained basement membrane (arrow). Early and late stage POF accumulate (top right panel) throughout spawning (ovary section Mat 2). The ovary tunica (T) is clearly much thinner in ovary section from female Imm (left middle panel) compared to female Mat 3 (right middle panel) respectively that also contain POF aged 45 or more days post spawning (arrows). The lower two panels show examples of encapsulated follicle cysts (EC) comprising the residual chorion (C), yolk granules (YG arrow) and POF > 150 days old (arrows) from ovaries in wild mature post spawning fish (Table 3). The scale bar = 1000µm.

Fig 4

Upper three panels: Cumulative production of spawned eggs from Atlantic cod (*Gadus morhua*) Mat 1 -3 in Experiment 2. The dates when biopsy samples were removed to study post ovulatory follicle (POF) production and when the fish were killed are indicated by vertical lines along the time axis. Lower three panels: Numbers of residual follicles classified as normal (black bars) hydrated (grey bars) and atretic (white bars) found in the ovaries of Mat 1 – 3.

Fig. 5

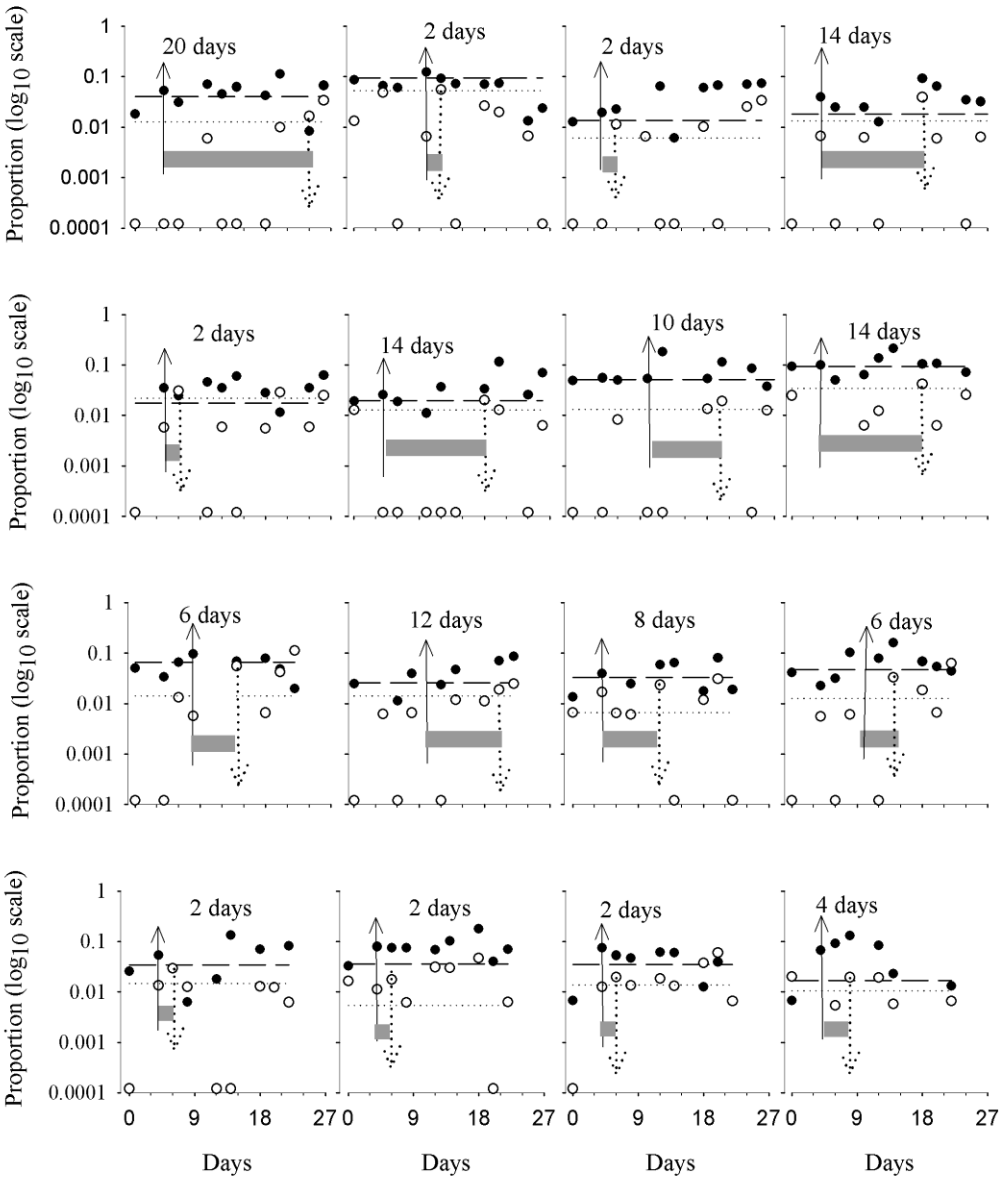
Temperature regime maintained during Experiment 3 lasting from 30 April until 12 August. The black and open circles refer to the North and Barents Sea simulations respectively whilst the triangles on the base line show when Atlantic cod (*Gadus morhua*) were sampled on Day 0, 14, 28, 56 and 104, respectively.

Fig. 6

Experiment 3: Area of leading post ovulatory follicle (POF) cohort measured in histological sections prepared from ovaries of Atlantic cod (*Gadus morhua*) kept in 5 m tanks simulating

(A) North Sea and (B) Barents Sea spring to early autumn warming regime sampled at internals from 30 April to 12 August. The solid lines where fitted with an exponential decay model y = a * exp (-b * day) using area specific coefficients shown in Table 4 and the dotted lines show ± 95% confidence limits. The filled data points apply to experimental fish kept in the North Sea (upper panel) and the Barents Sea (lower panel) and the open circle data points apply to wild fish (Table 3) collected in each area.

101 Fig 1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0.001
0.0001
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2 days
2 days
14 days
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14 days
10 days
14 days
6 days
12 days
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6 days
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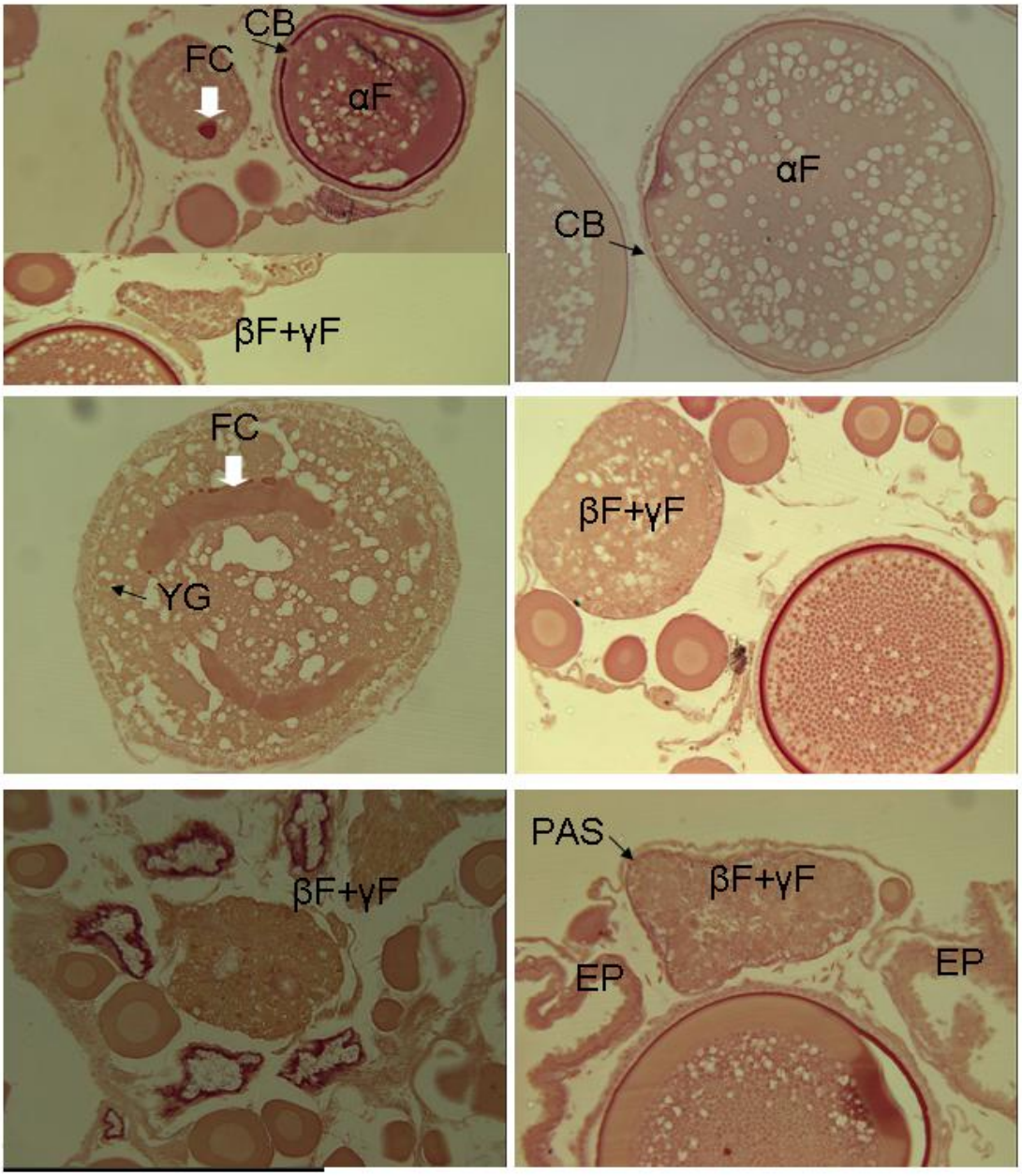
2 days
2 days
4 days
0
9
18
27
Days

102

103 Fig. 2



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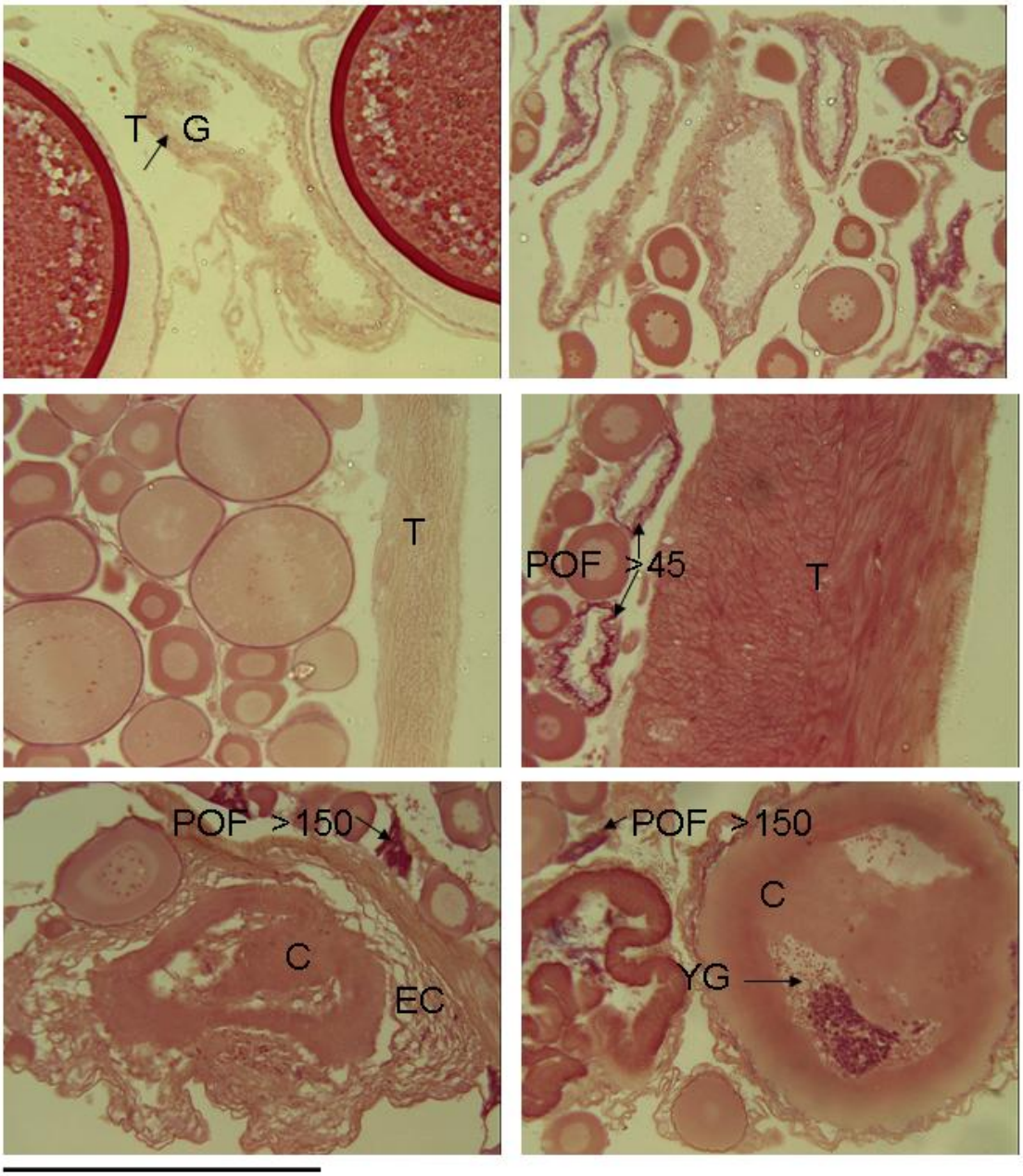
108 Fig. 3



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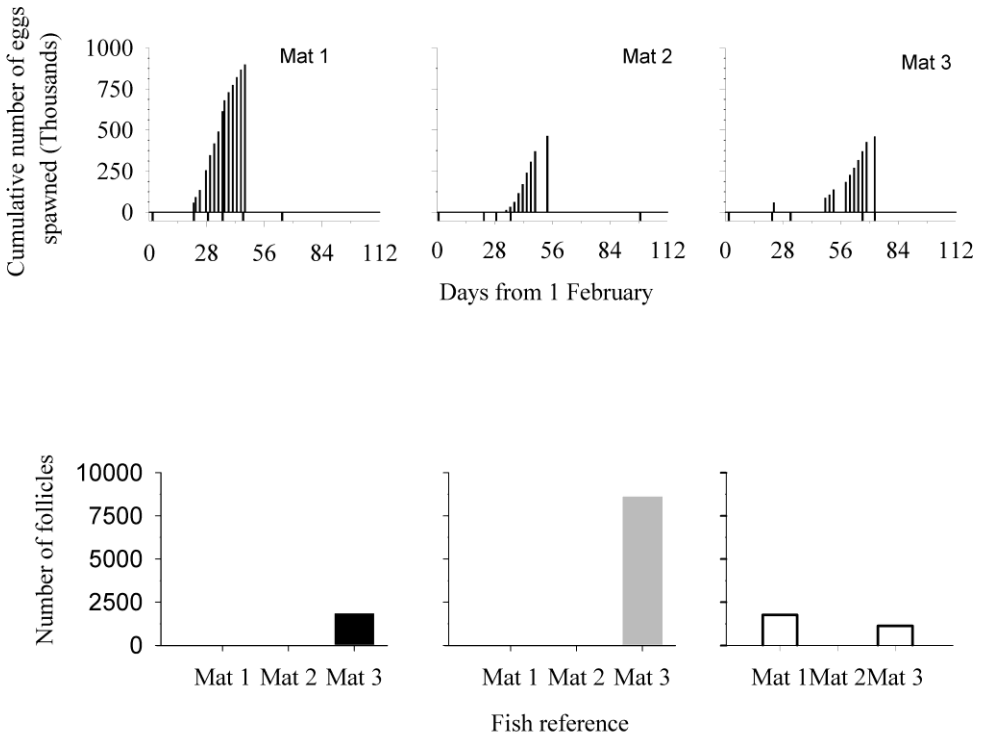
111 Fig. 4



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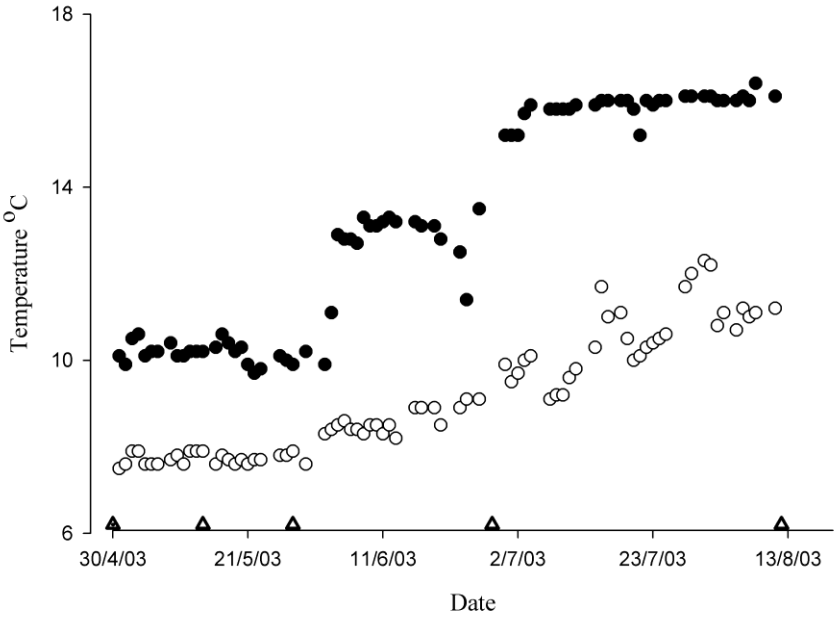
114 Fig. 5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10
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23/7/03
13/8/03
Date

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116 Fig. 6

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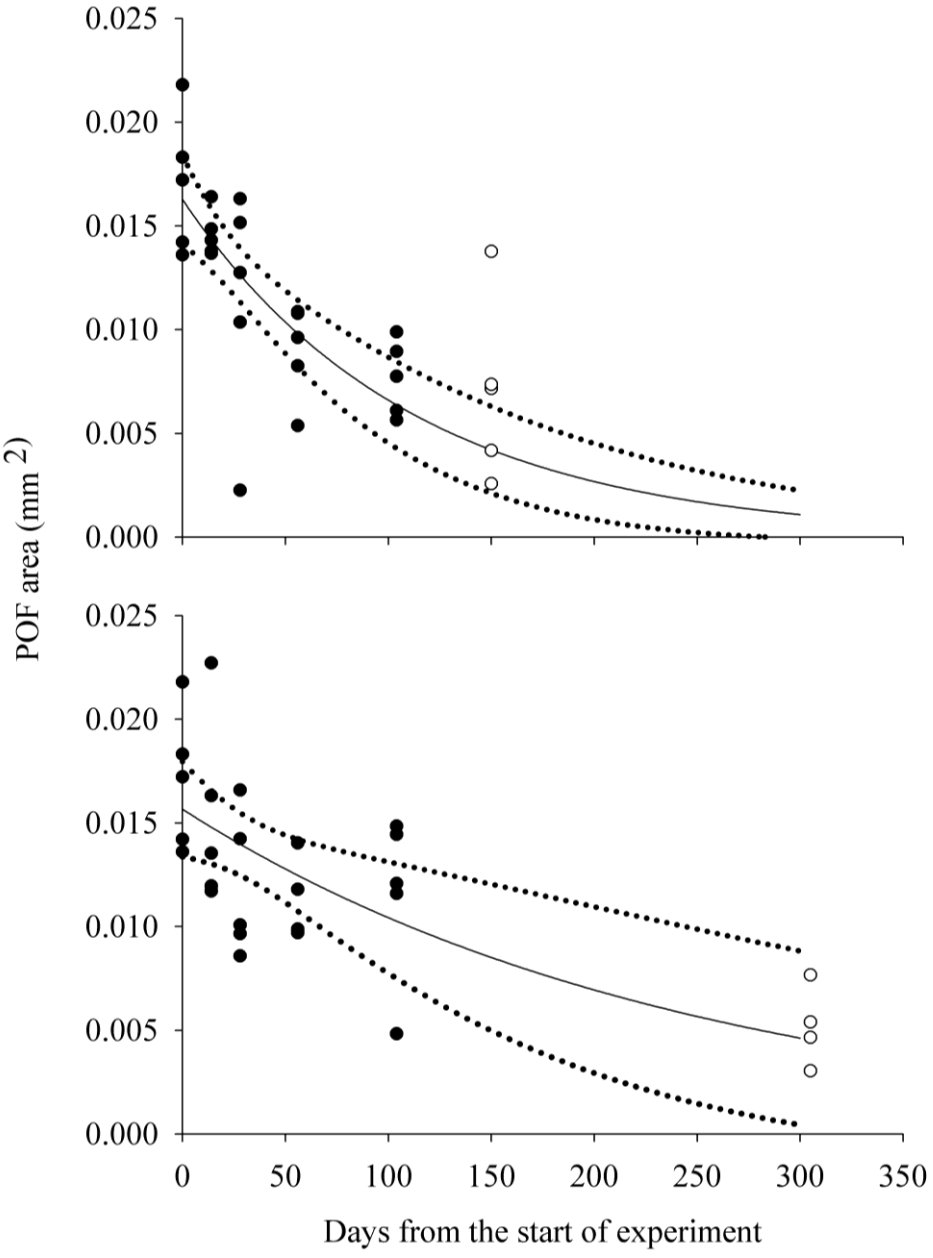

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