The application of tank experiments to the study of reproductive potential in teleosts using *Gadus morhua* as a test model

P.R.Witthames,, T.E. Andersen and O.S. Kjesbu

P.R.Witthames: Centre for the Environment Fisheries and Aquatic Science, Fisheries Laboratory, Pakefield Road, Lowestoft Suffolk NR330HT, England [tel: +1502 562244, fax: +1502 513865, email : p.r.witthames@cefas.co.uk]. T. E. Andersen and O.S. Kjesbu, Institute of Marine Research, P.O.Box 1870, N-5817 Bergen –Nordnes, Norway.
Key Words: Cod, tank experiments, fecundity, reproductive potential.

Age-based population assessments (VPA) have often failed to show a clear relationship between spawning stock biomass and either recruitment or biomass estimated from annual egg production. Recent work has shown that the inclusion of reproductive potential (a measurement of both the quality and quantity of eggs produced in relation to female size and age) helps to explain some of the variation in the stock and recruitment curve. Tank experiments will make it possible to develop tools to measure reproductive potential either directly from fecundity or indirectly from maternal reserves. This paper describes such experiments involving first year maturing cod (*Gadus morhua)*, in which egg production has been studied in relation to maternal reserves. Ovary biopsy samples were taken from the start until the end of spawning when the fish were killed in order to study the persistence of post-ovulatory and atretic follicles (the latter are oocytes that are resorbed during the vitellogenic phase) using histological methods. These results were examined to suggest ways to improve the detection of post spawning females (which is essential when making field assessments of maturity). Using this data it was also possible to quantify atresia in relation to the egg production cycle.

Introduction

A fundamental issue for fisheries science is the capacity of wild fish populations to sustain their numbers when facing heavy fishing mortality. This lowers age variability in the stock and adversely effects the stock and recruitment relationship (Marteinsdottir and Thorarinsson 1998) probably because of a strong interaction between fish size / age and reproductive

potential. In this paper reproductive potential refers to egg production in numerical terms rather than egg quality which could also be addressed by tank experiments (Solemdal 1997). Looking at egg production in numerical terms Northeast Arctic cod biomass, estimated by Virtual Population Analysis (VPA), was shown to be poorly correlated with population egg production (Marshall et al. 1998). The latter author (Marshall et al. 1999) also showed high reproductive potential (as total lipid energy) positively improved recruitment. More recently a fisheries independent assessment of cod, sole and plaice found spawning stock biomass (SSB) was 2.3, 2.7 and 4.3 times respectively greater than as indicated by VPA (Armstrong et al. in press).

This paper concerns the reproductive potential of captive cod starting with the relationship between potential annual fecundity of individuals and either their egg production or follicular atresia (death of developing eggs). The latter was quantified by taking tissue samples (biopsies) from sedated fish which subsequentially recovered to continue spawning. Spent ovaries were examined histologically to investigate the overall efficiency converting potential into realised fecundity and to develop criteria to identify post spawning from immature females. Finally the reserves of the fish were quantified using Fulton's condition factor (total fish weight / length^3) and related to atretic oocyte production to assess its role in regulating realised fecundity.

Materials and Methods

Fish and experimental protocols

We used fish reared from eggs spawned by Northeast Arctic cod in March 1997 as part of a mass rearing programme at Parisvatnet Norway (Otterå et al. 1999). After 15 months the fish were transported to the laboratory at Bergen where half were weighed to the nearest gram and their length measured to the nearest 0.5 cm. This data was used to estimate how much dry food (Felleskjopet: 'Cod pellet' , 10% fat, 53% protein, 18.5% carbohydrates) they would require for a ration size of 0.5% body weight per day. The fish were then divided into two groups that were kept separated by a net partition dividing a 30m^3 outdoor tank supplied by sea water pumped from about 100 metres depth from the adjoining fjord. Only one of these groups was used in this experiment and they were kept on these rations until the start of the experiment on January 27 1999 as 2 group fish. At this time the fish were anaesthetised with 5mg L^{-1} metomidate in oxygenated sea water (Mattson and Riple 1989; Thomas and Robertson 1991) followed by weighing and measuring total fish length. Also at this time a PIT

tag was inserted into the dorsal muscle just forward of the dorsal fin for subsequent identification and to remove a tissue sample (biopsy) from the gonad by catheterisation (McEvoy 1984; Kjesbu 1989). The gonad biopsy was examined to classify fish as sexually mature or sexually immature males and females. Further details on these fish and how they were allocated to each part of the experiment between the 1 and 2 February 1999 are shown in Table 1. Fish used to determine the reference potential fecundity were anaesthetised and killed by a blow to the head followed by removal of the ovaries which were then weighed to the nearest 0.1g.

Estimation of Potential fecundity in the reference group

A gravimetric method (Kjesbu et al. 1998) was used to estimate potential fecundity based on raising the number of vitellogenic oocytes (n = 565-1721) in subsamples (68 - 185 mg) viewed under a binocular microscope (x50 magnification) to the combined weight of both ovarian lobes (24 –120g). An important assumption in the method, that the vitellogenic oocytes were packed uniformly in both ovaries with respect to the minimum subsample weight, has been shown previously for this species (Kjesbu and Holm, 1994). A total of four subsamples were taken from the centre of the right ovary of each female and fixed in 4% formaldehyde buffered with 0.1M sodium phosphate pH 7.0 for preservation prior to counting the number of vitellogenic oocytes. In most cases the estimate of potential fecundity was based on only 2 subsamples. Three or four subsamples were counted in cases where the coefficient of variation for two or three subsamples was larger than 5%. The presence of hydrated oocytes in these gravimetric subsamples was also noted.

From each ovary a further sample was dehydrated and embedded in Historesin (Leica Norway) to prepare 5 µm sections that were stained by PAS Mallory Trichrome (Witthames and Greer Walker 1995). The sections were examined for signs that the fish had already started spawning as indicated by the presence of migratory stage nuclei, hydrated oocytes or post ovulatory follicles (POF) to ensure that no spawning fish were included in the estimate of potential fecundity (Hunter and Macewicz 1980; Lasker 1985).

Estimating potential fecundity for each experimental female

In each case the potential fecundity of the reference fish was divided by its length[3] and the mean of these values was raised by the length[3] of each experimental female to estimate its potential fecundity. This value was again weighted in proportion to the size of three large

batches collected in the middle of the batch production cycle relative to the mean of this value for all experimental fish.

Estimation and characterisation of realised fecundity

The ten experimental females (Table 1) were each placed with a mature male in one of the ten compartments (each female will be referred to as RT 1-10) of a 200m^3 indoor annual tank supplied continuously with fresh sea water from the adjoining fjord . A daily light cycle was maintained with artificial lighting. Temperature was recorded weekly in all compartments and the salinity varied between 34 and 34.5%. Following their introduction to the annual tank the ten pairs of fish were monitored daily noting their swimming activity and for signs of egg collecting in the surface seawater outflows. In three instances (Tank 1 9 Feb, Tank 2 18 Feb, Tank1 24 Feb) the males were not active and following the females (normal behaviour) so they were killed and replaced with active running males held in reserve. Food was not given from the start of the experiment until batch production ceased as cod do not normally feed during spawning (Kjesbu et al. 1991). Egg production from each female was collected as described in Kjesbu et al. 1991 and the diameter ($\pm$ 0.1 mm mean of 10 eggs), stage of egg development (Kjesbu 1989) and total volume of eggs was recorded for each batch produced. On one occasion the female in Tank 10 became unnaturally distended with ovulated eggs and these were removed by hand stripping. The mean diameter of these activated but unfertilised eggs was multiplied by 1.01 to give the mean fertilised egg diameter (Kjesbu et al. 1996). Further biopsies were taken from the ovaries (see above) at intervals during the spawning season (Fig. 1) following sedation of the experimental fish and processed to prepare stained resin sections as above. The structure of POFs was examined in relation to the time elapsed since spawning as determined by egg development stage.

Estimating atresia during spawning.

Alpha atretic follicles were identified in the resin sections following criteria described by Kjesbu et al. (1991) to estimate their abundance as a proportion of normal vitellogenic follicles. Ideally 200 such oocytes were scored in each sample but in some cases, when the ovary was near spent, the numbers were much lower. The number of atretic oocytes produced between each biopsy sample was calculated as in Table 2.

Observations on the post spawning ovary

The first experimental fish was killed on the 17 March and from then on, they were killed at intervals until the last fish died on the 10 May (Fig.1). Starting from the 7 April all the surviving fish were fed at the same rate as above. The ovaries were removed from each fish, weighed (±0.1g) and fixed for several weeks in phosphate-buffered formaldehyde. Subsequently cross sections 5 mm thick were cut out half way along the ovaries and embedded in Historesin to prepare resin sections which were stained as above in order to estimate the residual atretic and vitellogenic oocytes by a stereometric method (Emerson et al. 1990). In one case there were large numbers of vitellogenic and un-ovulated hydrated oocytes remaining and their numbers were quantified by the gravimetric method. Two of the immature fish were also killed on the 7 April and their ovaries processed as above to compare their histological structure with the spent ovaries. Measurements of ovary wall or tunica thickness and maximum previtellogenic oocyte diameter (repeated in 7 microscopic fields) were made using an Aphelion image analysis software with a resolution of 3.4 µm per pixel.

Results

Potential fecundity

All 21 females in the reference group were examined to estimate the mean potential fecundity condition (Fig. 2). Two fish were excluded because their ovaries contained many transparent to semi opaque oocytes of small size that were not clearly differentiated by size from the previtellogenic oocytes. Histological examination of the biopsies from the reference group indicated that no fish were close to final maturation (presence of migratory nuclei) or contained evidence that batch production had started (presence of hydrated oocytes or POFs). The potential fecundity condition in the remaining 19 fish was independent of fish length, as the slope was not significantly different from zero and the mean value was12.94 SD 3.6. This value was applied to predict potential fecundity in each experimental fish. The average relative potential fecundity was 1114 (SE 61.4) oocytes g^{-1} female weight.

Batch production in the experimental fish

Most egg batches contained high percentages of fertilised eggs (>50 %) except those from RT 4 and 7 where only 19 and 28 % respectively were fertilised (Fig. 3). These fish were excluded from the estimation of realised fecundity shown in Figure 1 because dead eggs

would tend to sink away from the surface net traps or from collection by the hand held nets. No eggs were produced by any of the spawning pairs in the first 10 days of the experiment (Fig. 1) confirming the histological evidence that the fecundity determined in the reference group was not negatively biased by inclusion of some spawning females. All experimental females commenced spawning between the 11 February and 5 March and produced regular batches on average for 24 days (range 17 to 32 days). Exceptionally females in RT 5 and 10 spawned over a much longer period (58 to 51 days respectively). The weighting values applied to the average reference female potential fecundity to predict individual fecundity of the experimental females were also calculated from this batch fecundity data (Fig. 3). Experimental fish were killed at intervals up to a maximum of 45 days (RT6) after producing their last batch and the number of residual oocytes estimated (Fig 4). Eight of the 10 experimental fish had less than 1% of their predicted potential fecundity remaining in the ovary. The female in RT 10 was killed very soon after producing a batch of eggs and probably had one more small batch of about 8000 eggs left to spawn. Only the female in RT 7 had substantial numbers of oocytes remaining in ovary and these too would probably also have been spawned after a short lapse in time had it not been killed. Overall it was clear that cod spawn, or remove by atresia all their potential fecundity during the annual egg production cycle and the 8 experimental females (Fig. 1) spawned 71% (SE 5%) of their predicted potential fecundity.

Comparing immature and spent ovaries and the persistence of POF

After less than 12 hours (estimated from the age of fertilised eggs), following spawning the first batch by RT9, the POF had collapsed from a thin band that use to lie round the hyaline oocyte (Fig. 5). It now took on the form of a hollow centred asymmetric curly band, 530 µm across its longest axis, of granulosa and thecal layers separated by a PAS positive basement membrane. The POF continued to shrink with age accompanied by more intense PAS staining that was still clearly present 45 days post spawning (RT6). It was also noted that larger previtellogenic oocytes were present in the two immature fish (up to 185 se 3 and 224 se 6 µm respectively) compared to 131 se 9 µm in the spent fish from RT 10 and RT6. The ovary tunica was much less developed (120 µm thick) in the immature fish and up to 650 µm in the ovary 45 days post spawning.

Estimation of total alpha atretic oocyte production

The two experimental fish (RT4 and 7), excluded from the above analysis because of low batch fertilisation, did not appear particularly different in the proportions of alpha atretic oocytes seen in their biopsy samples. Expressed as a percentage of residual fecundity alpha atretic oocytes peaked in both these fish at less than 5% (RT7) compared to a maximum of 9.8 % in RT8 for the other 8 fish.   In all 10 experimental fish the numbers of atretic oocytes tended to increase most during the early part of spawning (Fig. 1). Total production of alpha atretic oocytes was very variable between the eight experimental fish and was related to their pre-spawning condition (Fig. 6). It was possible to estimate the duration of the alpha atretic stage using the method in Table 2 and the average for all 8 experimental fish was 8.2 days (SE 4.8) with values ranging from a minimum of 0.44 to a maximum of 40.3 days.

Discussion

Considerable variability was found in our estimate of atresia stage duration and this probably resides in the large CV (27.9%) in the mean potential fecundity applied to individuals in the experimental group. Since this work was carried out high frequency ultra sound has been used to estimate potential fecundity with good precision (Karlsen and Holm 1994) and this should be used in further studies to improve the CV of alpha atresia stage duration. Although instantaneous estimates of atresia in wild cod are low (Kjesbu et al. 1998) the rapid turnover would imply a large reduction in potential fecundity which would have a direct consequence of raising SSB when applied in egg production based stock assessment (Armstrong 1995). Viewed from the current ICES session theme experimental laboratory studies have three types of application to fisheries science. The first use is to provide a model for studying the dynamics of egg production and atresia for stock assessment e.g. cod, mackerel, sole horse mackerel and plaice (Armstrong 1995; ICES 1999). Secondly to develop tools to differentiate immature from post spawning females for use in maturity assessment. Finally; to improve fisheries assessment by incorporating environmental (temperature) and biological effects (food availability) on reproductive potential into predictive multispecies models including how migration fits into their life cycle strategy.

References

Armstrong, M.J., 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., and Woolner, L., In press. 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.

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

Hunter, J.R. and Macewicz, B.J., 1980. Sexual Maturity, Batch Fecundity, Spawning Frequency, and Temporal Pattern of Spawning for the Northern Anchovy, Engraulis mordax during the 1979 Spawning Season. CalCofi 21:139-149.

ICES 1999 / G:5 Ref. :D. Report of the working group on mackerel and horse mackerel egg surveys.

Karlsen, Ø., Holm, J.C. 1994. Ultrasonography, a non-invasive method for sex determination in cod (*Gadus morhua*). J. Fish Biol.. 44: 965-971.

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

Kjesbu O.S., Klungsøyr, J., Kryvi, H., Witthames, P.R., & Greer Walker, M., 1991. Fecundity, atresia and egg size of captive Atlantic cod (*Gadus morhua*) in relation to proximate body composition. Can. J. Fish. Aqua. Sci. 48:2333-2343.

Kjesbu, O.S., Holm, J.C., 1994. Oocyte recruitment in first time spawning Atlantic cod (*Gadus morhua*) in relation to feeding regime. Can. J. Aqua. Sci. 51: 1893-1898.

Kjesbu, O.S, Solemdal, P., Bratland, P. & Fonn, M., 1996. Variation in annual egg production in individual captive Atlantic cod (*Gadus morhua*). J.Fish. Aqua. Sci. 53:610-620.

Kjesbu, O.S, Witthames, P.R., Solemdal, P., & Greer Walker, M., 1998. Temporal variations in the fecundity of Arcto Norwegian cod (*Gadus morhua*) in response to natural changes in food and temperature. J. Sea Res. 40:303-321.

Lasker, N.C.H., 1985 An egg production method for estimating spawning biomass of pelagic fish: application to the northern anchovy, *Engraulis mordax*. NOAA Tech R NMFS 36:43-50.

Marshall, T.C., Kjesbu, O.S., Yaragina, N.A., Solemdal, P., and Ultang, Ø., 1998. Is spawning stock biomass a sensitive measure of the reproductive potential of Northeast Arctic cod. Can.J.Fish.Aquat.Sci. 55(7):1766-1783.

Marshall, T.C., Yaragina, N.A., Lambert, Y., and Kjesbu, O.S., 1999. Total lipid energy as a proxy for total egg production by fish stocks. Nature 402:288-290.

Marteinsdottir, G. and Thorarinsson, K., 1998. Improving the stock and recruitment relationship in Icelandic cod (Gadus morhua L.) by including age diversity of spawners. Can. J. Fish. Aquat. Sci. 55:1372-1377.

Mattson, N.S., Riple, T.H., 1989. Metomidate, a better anaesthetic for cod (*Gadus morhua* in comparison with benzocaine, MS-222, chlorobutanol, and phenoxyethanol. Aquaculture 83: 89-94.

Mcevoy, L. (Barton), L.-A. 1984. Ovulatory rhythm and over-ripening of eggs in cultivated turbot, *Scopthalmus maximus* L. J. Fish Biol.24: 437-448.

Otterå, H., Jørstad, K.E., Svåsand, T. & Kristiansen, T.S. 1999. Migration patterns and recapture rates of North-east Arctic and Norwegian coastal cod reared and released under similar conditions. J. Fish Biol. 54: 213-217.

Solemdal, P., 1997. Maternal effects – A link between the past and the future. J. Sea Res. 37:213-227.

Thomas, P., Robertson, L., 1991. Plasma cortisol and glucose stress responses of red drum (*Sciaenops ocellatus*) to handling and shallow water stressors and anaethsia with MS-222, quinaldine, sulphate, and metomidate. Aquaculture 96: 69-86.

Witthames, P.R. and Greer Walker, M. 1995. Determination of fecundity and oocyte atresia in sole (*Solea solea* L.) (Pisces) from the Channel, the North Sea and the Irish Sea. Aquat.Living.Resour. 8(1): 91-109.

Table 1
Details of the number of fish by sex used in each part of the experiment including their mean length.

Use in the experiment	Female		Male	
	Number of fish	Mean length cm (std)	Number of fish	Mean length cm (std)
'Reference' – for potential fecundity	21	40.0 (3.0)		
'Experimental' – for realised fecundity	10	39.0 (0.9)	10	40.4 (2.3)
Reserve			29	37.4 (3.6)
mature	7	38.7 (1.8)		
Immature	3	36.0 (5.5)	3	33.3 (5.8)

Table 2
An example, taken from the fish in Round Tank 3, of the method used to calculate the duration of the alpha atretic stage.

Potential fecundity weighted by batch size (P_f)= 776450						
				Production in period of:		
Date of biopsy or final ovary sample	Interval between biopsy samples (I_{days})	Proportion of residual fecundity atretic (R_{pa})	Standing stock of atretic oocytes (A_s)	Eggs spawned (E_p)	Alpha atretic oocytes (A_p)	Residual Fecundity (R_f)
1 February		0.0098	7612			768839
16 February	15	0		130011	83307	555521
22 February	6	0.0010		129138	23591	402793
1 March	7	0.0968		115130	207582	80082
8 March	7	0		36902	33603	9577
17-Mar-99 killed	9	1	1393	0	9577	0
Estimated duration of alpha atretic stage A_d = 0.621 (days)						

Parameters calculated.

P_f Weighted mean potential fecundity in the reference sample. The weighting value for each experimental fish was based on the sum of three egg batches in the middle of spawning relative to the average for the experimental group shown in Figure3.

A_s Start of experiment: $R_{pa} \cdot P_f$
End of experiment: Determined in the ovary by sterometric analysis

(A_p) $R_{f-1} - E_p/2 \cdot R_{pa\ mean} \cdot I_{days} \cdot A_d$ where R_{f-1} is the residual fecundity at the end of the previous period and R_{pa} mean is the average of R_{pa} values from the start and finish of I_{days}.

R_f $R_{f-1} - E_p - A_p$

A_d Values where adjusted until the final value of R_f was either zero or equal to the number of residual vitellogenic oocytes determined in the ovary by stereometric analysis.

Figure legends

Figure 1
Cumulative production of spawned eggs and atretic oocytes during the experiment in Round tanks 1-3,5-6 and 8-10. The removal of biopsy samples to estimate atresia and when the fish were killed are indicated as bars below 0 on the time axis.

Figure 2
Potential fecundity condition determined by the gravimetric technique in the reference group, n=19. The r^2 of the fitted regression was 0.02 and the slope was not significantly different from zero. The 95% confidence limits around the mean length and fecundity condition are shown by vertical and horizontal error bars.

Figure 3
Details of each female's length (cm), the mean proportion of eggs fertilised over the spawning season and her batch fecundity production observed in the Round Tank (RT) 1-10. The dark bars show which batches were used to weight (values shown in each panel) the mean potential fecundity of the reference group to predict potential fecundity in each female in the experimental group.

Figure 4
Estimates of the residual fecundity classified by histological analysis into numbers of vitellogenic (top panel) hydrated (middle panel) alpha atretic oocytes (lower panel) found in the ovaries of the experimental fish at the end of the experiment.

Figure 5
Histological sections stained by PAS and Mallory's trichrome. Panel 1: RT9 biopsy taken on 22 Feb 12 hours after spawning showing a post ovulatory follicle (P24) with a large lumen and a PAS positive basement membrane (a fine line between the follicle layers). Panel 2: RT10 ovary section (killed with one batch prior to ovulation remaining) showing hydrated oocytes (H), older POF with collapsed lumen (P24+) and an ovary tunica (OT) 400µm or more wide. Panel 3: RT6 ovary section, killed 45 days post spawning with old POF (Po) still clearly visible with PAS staining and the ovary tunica up to 650 µm wide. The previtellogenic oocytes (PVO max) were 131µm or less in diameter. Panel 4: Ovary section of an immature female ovary killed on the 7 April showing a thinner ovary tunica (<120 µm) and larger PVOmax (224 µm) compared to RT10 and RT6.

Figure 6
Estimates of alpha atretic oocyte production expressed as atresia condition (A_c =Number of atretic oocytes / length ^{3}) in the experimental fish (excluding RT4 and 7) in relation to their prespawning condition (P_c). The line fitted from the formula $A_c = -21.0 P_c + 28.5$ n=8 explains 54% of the variance between the two variables.
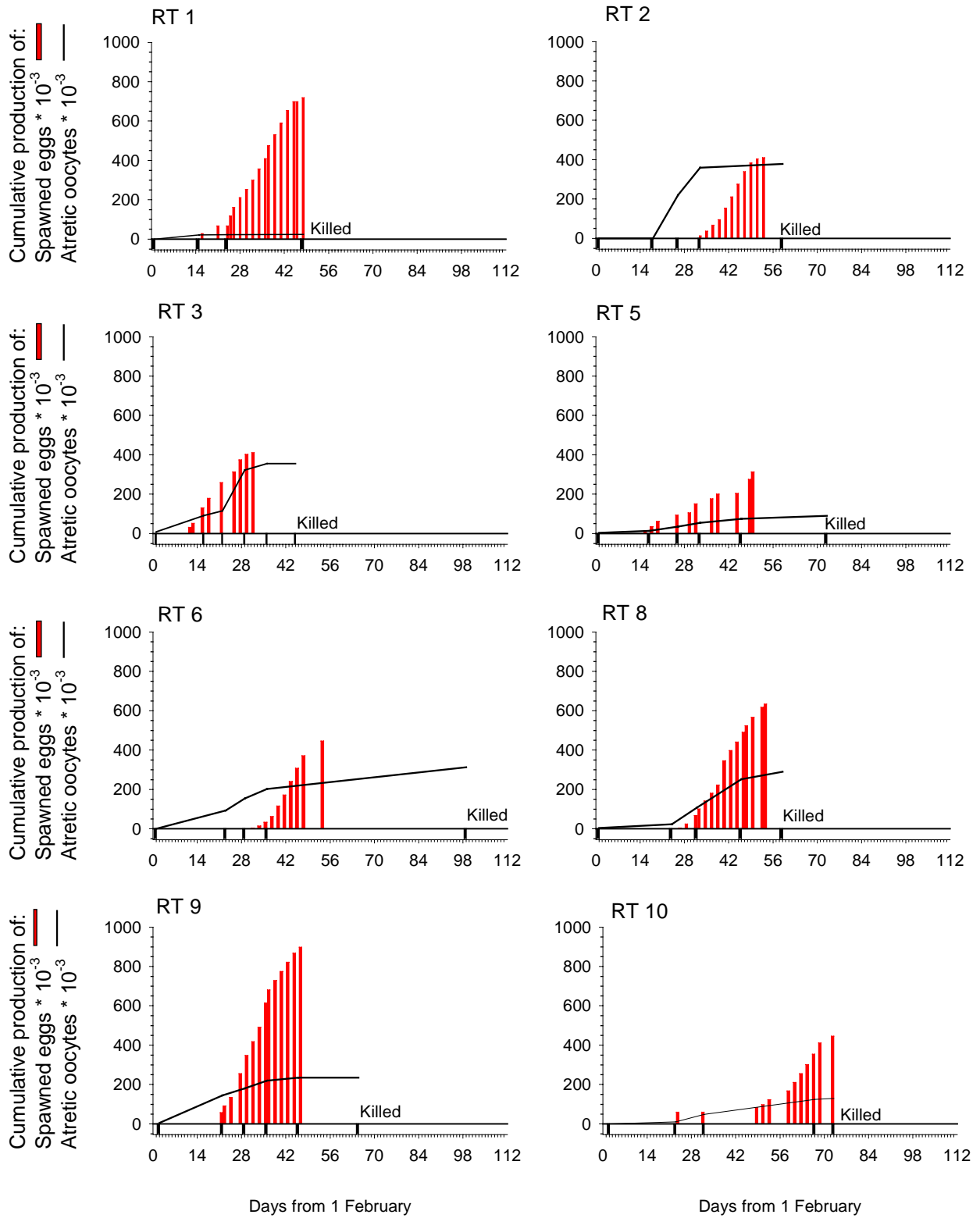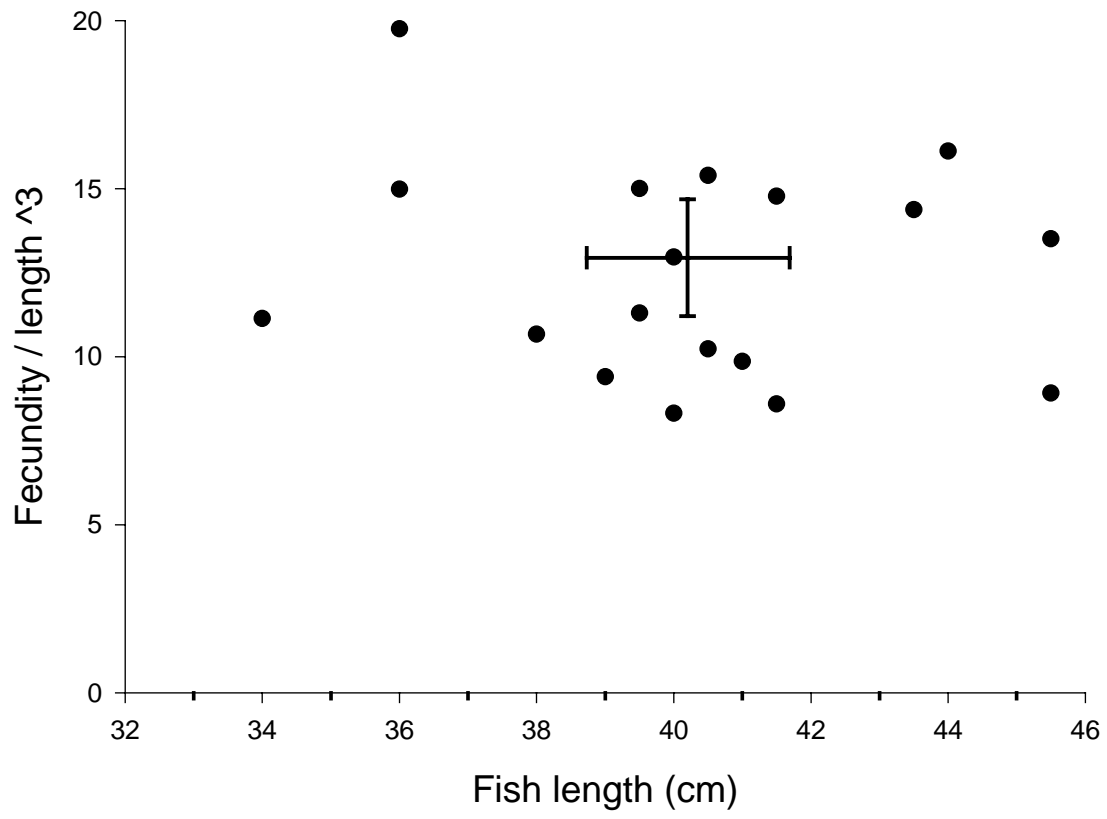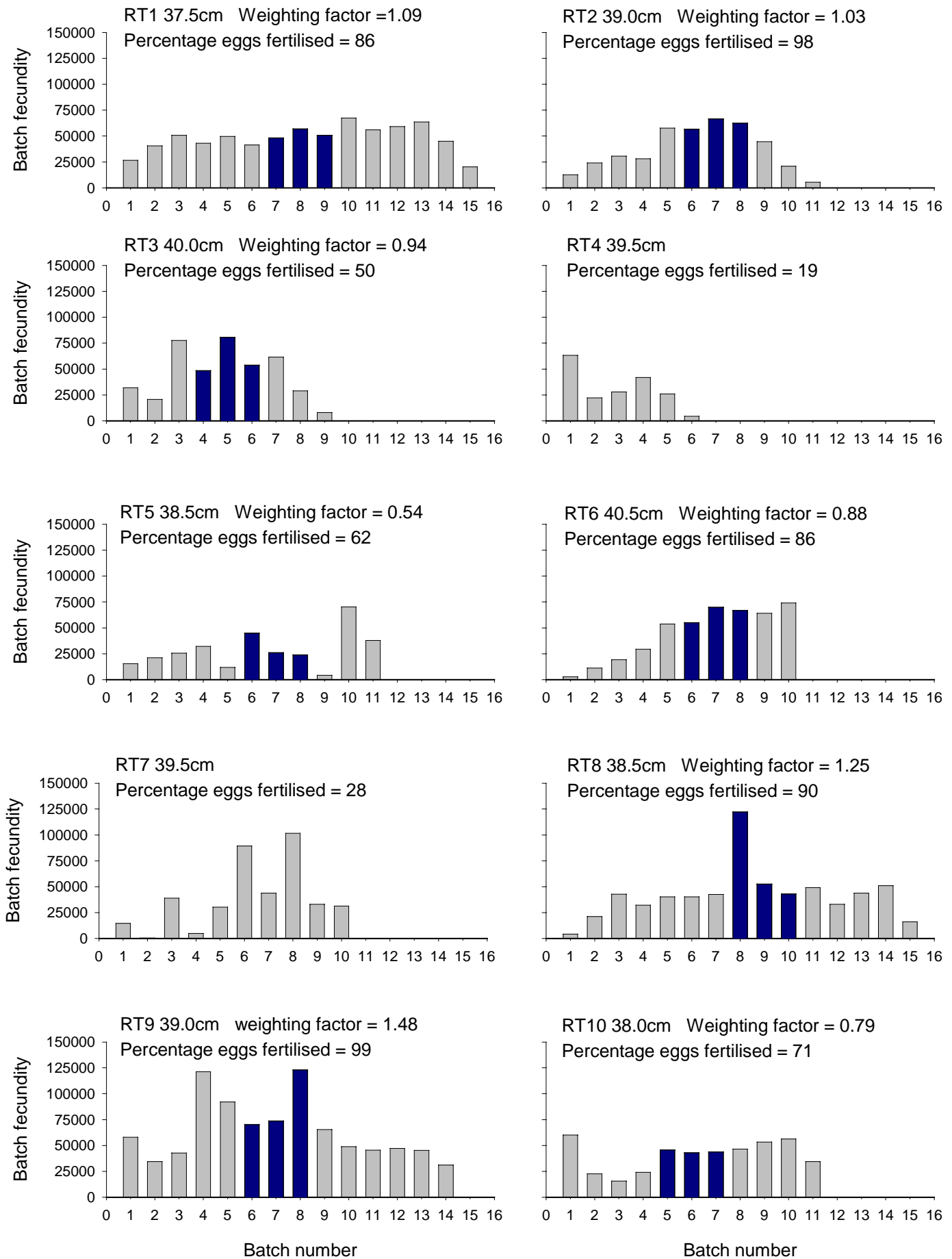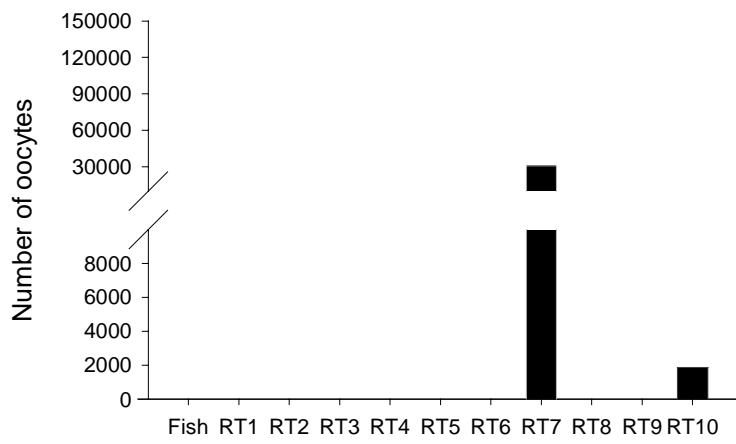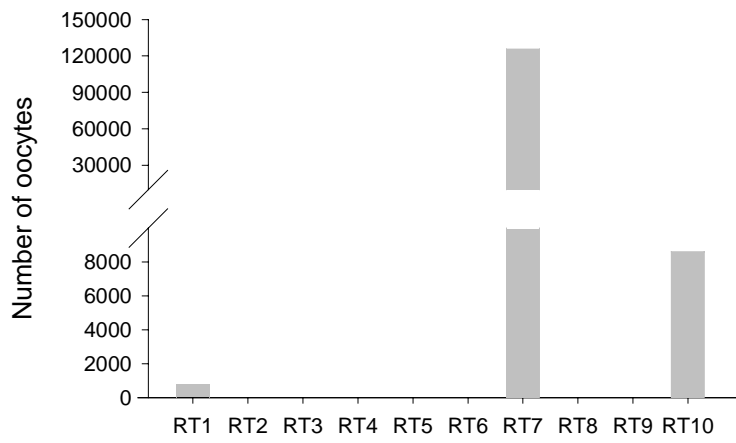
Fig. 1

Fig. 2

Fig. 3

Fig. 4

Panel 1

Panel 2

Panel 3

Experimental fish reference

Fig. 5

Panel 1

P24 >

Panel 2

< O T >

H >

P24+ >
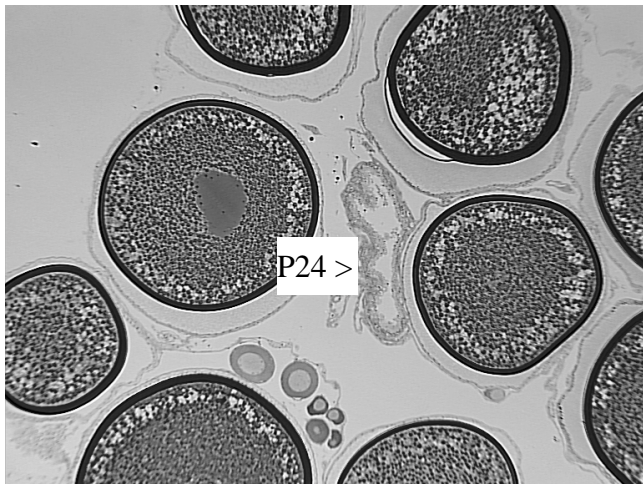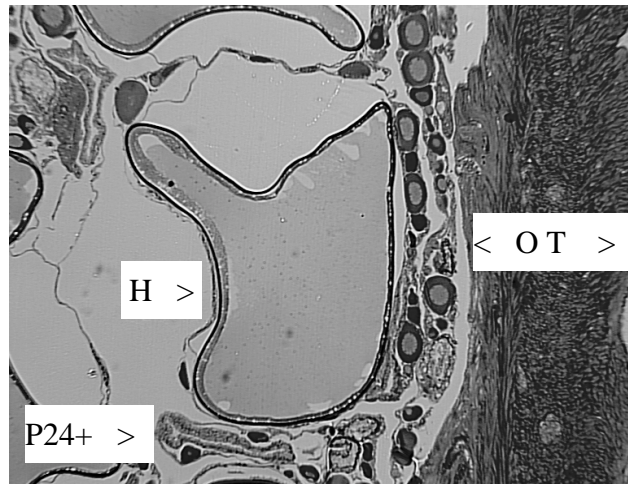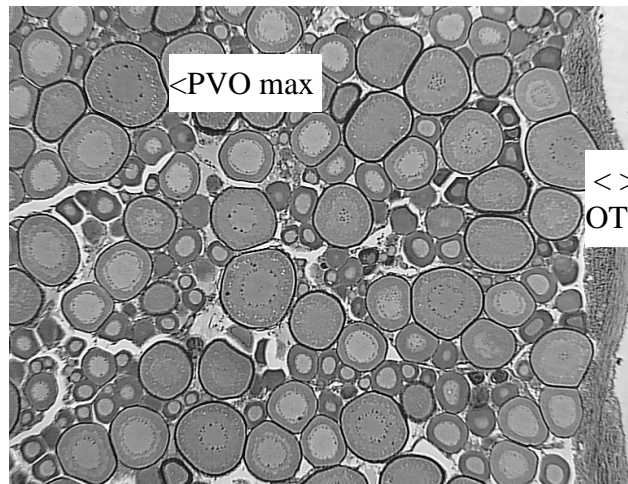
Panel 3

<Po

<PVO max

< O T >

Panel 4

<PVO max

< >
OT

1 mm

Fig. 6