REPORT ON GRAYLING RESEARCH AND PRODUCTION IN THE ANGLIAN REGION OF THE NATIONAL RIVERS AUTHORITY DECEMBER 1988 TO APRIL 1989

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1. INTRODUCTION

This report is concerned with the second year of research and production of grayling (Thymallus thymallus) by staff of the Fish Production Unit, based at the Regional Fisheries Laboratory, Brampton. These studies, in collaboration with the Yorkshire Region NRA unit, follow on from work performed in the pre-spawning season of 1988. As a preface to this report, a brief summary of the aims, approaches, results and conclusions from the 1988 report are presented. (This earlier work has been reported in detail by Brighty and Jordan (1988)).

1.1 Research performed in 1988

The initial work involved the establishment of a captive broodstock population in tank facilities at Brampton and at Costessey Fish Farm. Wild brood fish were obtained from the R. Kennet and were introduced into tank systems at Brampton and Costessey at the end of January 1988, some two and a half months before the expected time of spawning. Within three weeks of being placed into the tanks, despite feeding well on maggots, the fish began to show signs of a bacterial and secondary fungal infections. Treatment with Terramycin to counteract the furunculosis infection and regular prophylactic malachite green baths, ensured that the loss of brood fish was small. However, analysis of the female gonads revealed that regression of the oocytes has been initiated, represented by a loss of turgor within the oocyte and agglutination of yolk without the breakdown of the germinal vesicle. An attempt at induced spawning was made using the methodology published by Kouril and Barth, (1987), but no ovulations were achieved (see later). Thus, either the stress of capture, handling and introduction into an artificial tank environment, compounded by bacterial and fungal infections, or the anti-biotic treatment of Terramycin had effected oocyte resorption. These fish were all transferred to Brampton where they were maintained for the remainder of the year on a diet of maggots, some natural food and pelletted fish food and began to adapt well to tank conditions. These fish were available for induced spawning in the 1989 spawning season. From this part of the project, several important points were determined. Primarily, it was clearly demonstrated that grayling are very susceptible to handling, requiring regular malachite green treatments to prevent fungal growth, and only if absolutely necessary should Terramycin be administered to broodfish as this may cause oocyte regression. The attempt at induction of

ovulations, using the published methodology was unsuccessful, but this was thought to due to oocyte regression.

The second part of the project was to use wild broodfish for induced spawning purposes. The absence of any suitable grayling populations in the Anglian region for this purpose necessitated the use of large population in Yorkshire, in the West Beck, at Great Driffield. In cooperation with Yorkshire Region NRA unit, mixed groups of grayling were electrofished from the river at intervals during the pre-spawning season, from mid-February to early-April. At each sampling occasion, mature, wild broodstock were taken to the Regional Fisheries Laboratory at Brampton for spawning induction experiments. The first part of the programme was to determine whether the fish were in a suitable state of maturity, with respect to oocyte size, germinal vesicle position within the oocyte and external characteristics, for attempts at artificial hormonal induction of ovulations. In the first sample, taken in February, the oocytes did not appear to be fully developed with the germinal vesicles being eccentric but not peripheral. However, the second sample of grayling, taken in early March, revealed that final maturation had progressed development and a batch of fish was taken for spawning experiments.

This first spawning trial followed two established methodologies developed by Czechoslovakian scientists. A carp pituitary extract (CPE) injection was given to some brood female grayling whilst treatment of the remainder was with an analogue of luteinising hormone-releasing hormone (LHRH-A) and dopamine antagonist, Pimozide. Neither treatment caused ovulation in the broodfish, but in most, some oocytes regression had occurred. It was postulated that the hormonal dose was therefore too high. Over the next five weeks, four samples of brood grayling were taken for spawning induction. The aim was, using CPE only, to reduce sequentially the dose level until the appropriate physiological responses were observed, i.e. promotion of germinal vesicle position, germinal vesicle breakdown (GVBD) and ovulation, and not oocyte regression. In the last hormonal induction experiment, an appropriate CPE dose was determined, 0.1 mg kg^{-1}, to promote germinal vesicle migration (GVM) and GVBD, representing 1/50th of the original dose advocated by the published methodologies. No ovulations were achieved however after further hormonal injections. A final experiment was applied to some broodstock from the River Nidd, North Yorkshire. These fish were electrofished off the spawning beds and any ovulating females were handstripped on the bank, fertilised and taken back to hatcheries in Yorkshire and Brampton. The success was mixed and data were compared between both hatcheries. More of the oocytes fertilised in Woynarovich fertilisation solution were fertile than those fertilised in river water. This implied that sperm life was prolonged in the Woynarovich solution and

should be adopted as a routine protocol for grayling. However, oocytes transported for four hours to Brampton exhibited a low overall survival level, suggesting that some damage had occurred in transit. Some fry were however reared at Brampton from this batch.

There are several important conclusions from the 1988 experiments. Firstly, the background physiology of the grayling was elucidated during the pre-spawning period. A technique to remove gonad tissue, ovarian biopsy, was adapted for the much larger grayling oocytes and data on the progression of GVM were recorded. However, more specifically, there were no clear indicators of pre-spawning readiness and this point was still perhaps the most important, even before spawning induction was attempted. Other practical problems were solved, including the setting up of induced spawning trials, and the short-term care and recovery of diseased fish. The induced spawning trials demonstrated that CPE was not effective in inducing ovulations under the experimental conditions used, and only in very low concentrations was GVM and GVBD effected in some, but not all broodfish. The hormone was ineffective probably because of poor activity or quality, or, more likely, the phylogenetic difference between the gonadotropin hormone (GtH) in the CPE and the endogenously synthesised grayling GtH was too great for CPE to be effective. The health of the wild broodstock during the experiments was very good and possible adverse effects of stress can be discounted as all injections were generally given within four hours of capture. Some progeny from the hand-stripped grayling were reared, but a continual problem with systemic bacterial infections caused a high mortality of fry during the summer months. Rearing methodologies were developed for the first feeding and growing-on of fry by both Anglian and Yorkshire NRA Regions.

In conclusion, the experiments performed in 1988 demonstrated that there was still a need for a reliable induced spawning methodology. The hormone did not perform as expected, but a similar hormone was available which is more phylogenetically compatible. Further research was also needed to establish the appropriate time to administer hormones to induce ovulations and this would be the first priority for the studies in 1989. The feeding trials performed by the staff of Yorkshire NRA were successful but further work would be necessary to ensure that fry rearing was possible over a two-year period.

2 EXPERIMENTS TO DETERMINE PRE-SPAWNING READINESS IN GRAYLING

The results obtained from the induction of spawning experiments in 1988 revealed that 'normal' pituitary extract doses caused non-viability of the oocytes. Sequential reduction of the dose level, to 1/50th of the original amount

administered, was successful in completing germinal vesicle migration but not ovulation. The following series of experiments were designed to investigate the *in vitro* effects of salmonid pituitary extract (SPE) on final maturation and ovulation of grayling oocytes. It was hypothesised that the effects of gonadotropin treatment observed in previous experiments *in vivo*, i.e. non viability, should be reproducible *in vitro*. This would therefore reveal the likely pituitary extract dose range at which non-viability occurs and enable a suitable dose level, sufficient to stimulate GVM, GVBD and ovulation, to be determined. Essentially, the *in vitro* incubation experiments involve the immersion of oocytes in a buffered ringer, containing a known quantity of pituitary extract. A range will reveal the dose dependency of the treatments. The desired effects of gonadotropin stimulation involve the promotion of GVM, the initiation and completion of GVBD and ovulation. These effects have been shown in experiments with salmonid species (Kagawa, Young and Nagahama, 1982; review: Nagahama, 1983), but with cyprinids, the completion of GVM or initiation of GVBD is the end observation (Kime and Bieniarz, 1987; Kime *et al.*, 1987). This is largely due to the death of the oocyte at the higher incubation temperatures of these particular experiments.

2.1 Experiment 1 7th December 1988

This first experiment was performed on grayling obtained from the West Beck, Great Driffield, at the time close to the completion of oocyte growth. Vitellogenesis appears to be progressing at this time as the oocytes had not reached pre-spawning size. Thus, this experiment is an appropriate starting point to follow GVM from its initiation in the pre-spawning period.

Materials and Methods

Animals

6 female grayling were electrofished by personnel from Yorkshire NRA and Anglian NRA from the West Beck, the same study site as earlier *in vivo* experiments, on 7 December 1988. The fish were transported to the Regional Fisheries Laboratory, Brampton for subsequent experimental use.

Materials

A stock solution of salmonid pituitary extract (Argent, USA) of 1 mg ml^{-1} in phosphate buffered saline (pH 7.5) was prepared. Serial 10 fold dilutions of the stock in PBS were made to form stocks from 0.01 to 100 ug ml^{-1}. Tissue culture trays, each containing 12 chambers, were used for the incubations. 2 ml of each of the five stock solutions were

aliquotted out in duplicate into separate chambers, and 2 ml of PBS, in duplicate, was pipetted into the remaining two chambers.

Methods

Three female grayling were killed and somatic analyses recorded. 100 oocytes were cleared in a clearing solution of 65 % glacial acetic acid in IMS, and the positions of the germinal vesicles classified. A mean oocyte diameter was determined for each fish and a plasma sample was taken for ovarian steroid analysis. The ovaries were dissected out and groups of 30 oocytes were placed in each of the chambers in the tissue culture trays. Four trays were prepared for each fish used in the experiment, a tray being taken at one of the four time intervals during the experiment, 8, 16, 24 and 48 hours for analysis. The trays were placed in a chest incubator, maintained at a temperature of 10 °C. At each of the four time intervals, a tray from each female grayling was analysed. 1 ml of PBS was removed from each chamber and stored at -20 °C, and the remainder discarded. Clearing solution was added to the oocytes in each chamber and the position of the germinal vesicle noted after clearing. Oocytes were discarded after analysis.

Results

The results of the initial somatic analyses are given in Table 1. The data show clearly that the oocytes had not attained the pre-ovulatory size, compared to data from the first group of experiments in 1988. Similarly the position of the germinal vesicles in oocytes taken before incubation were all at stage 2 or 3. The results of the effects of gonadotropin (GtH) on grayling oocytes are presented in Tables 2 to 4. Little change in GV position occurred during incubation at all SPE concentrations and after up to 48 hours. The PBS samples taken from the chambers during incubation were analysed for ovarian steroids to reveal the steroidogenic activity of the oocyte follicles after GtH stimulation.

Discussion

It is clear that the oocytes had not attained the usual pre-spawning size by early December and it is likely therefore that vitellogenesis had not been completed. The plasma samples should confirm this if levels of the vitellogenic steroid 17β-estradiol (17β-E_2) are high. Clearly, if vitellogenesis is still proceeding, then the processes of final maturation would not occur until oocyte growth had been virtually completed. If this is the case, then it would explain the apparent little change in GV position after *in vitro* stimulation with GtH. Steroid analyses of the

Table 1 Somatic analyses taken of female grayling used in the in vitro experiment December 1988

Female	Length (cm)	Weight (g)	Ovary Weight (g) (BW%)	Mean Oocyte Diameter (mm)	G V Position (%)	
					2	3
1	29.7	361	28 (8 %)	2.06	71	29
2	31.5	366	29 (8 %)	2.15	62	38
3	30.2	381	27 (7 %)	2.08	66	34

Table 2 Germinal vesicle position in grayling oocytes during in vitro incubation with salmon pituitary extract

Female 1

Time (hours)	8		16		24		48	
GV	2	3	2	3	2	3	2	3
SPE (ug ml^{-1}) 0	58	42	53	47	53	47	49	51
0.01	35	65	25	75	42	58	40	60
0.1	46	54	33	67	38	62	41	59
1.0	60	40	40	60	38	62	41	59
10.0	39	61	32	68	35	65	33	67
100.0	42	58	32	68	24	76	38	62

Table 3 Germinal vesicle position in grayling oocytes during in vitro incubation with salmon pituitary extract

Female 2

Time (hours)	8		16		24		48		
GV	2	3	2	3	2	3	2	3	
SPE (ug ml^{-1}) 0	57	43	41	59	47	53	36	64	
							2	3	3+
0.01	45	55	30	70	35	65	32	46	22
							2	3	
0.1	44	56	32	68	53	47	38	62	
1.0	40	60	24	76	38	62	38	62	
10.0	52	48	33	67	53	47	33	67	
100.0	47	53	31	69	38	62	33	67	

Table 4 Germinal vesicle position in grayling oocytes during in vitro incubation with salmon pituitary extract

Female 3

Time (hours)	8		16		24		48	
GV	2	3	2	3	2	3	2	3
SPE (ug ml^{-1}) 0	63	37	42	58	52	48	42	58
0.01	60	40	29	71	52	48	51	49
0.1	46	54	25	75	45	55	46	54
1.0	48	52	36	64	55	45	53	47
10.0	42	58	27	73	49	51	44	56
100.0	45	55	32	68	49	51	50	50

phosphate buffer used to incubate the oocytes should provide information of the steroidogenic activity of the oocyte follicles. It is anticipated that 17β-E_2 will be high in the buffer, and probably also testosterone, its precursor.

It is interesting to note that the germinal vesicles in half of the oocytes were eccentric in position, even though it appears that oocyte growth had not been completed. It is possible that as the oocyte develops over a certain size, around 1.8 mm, the germinal vesicle assumes an eccentric position as the oocyte enlarges further, such that the vesicle is at a fixed distance from the micropyle. If there is an absence of any maturing hormones in the incubation media and plasma, then it would confirm that germinal vesicle migration had not been initiated and the eccentric position of the GV was an artifact. The implication for future experiments would be to read the GV position 2 and 3 as the same position and stage 3+ as a vesicle undergoing migration. A further point regarding the assessment of germinal vesicle position is the subjective nature of all observations. The continuity of readings is essential as demonstrated by the observations taken during the experiment. One observer took readings at 8, 24 and 48 hours whilst the readings at 16 hours were performed by another. The differences in staging was very clear (Tables 2, 3 and 4).

Other problems encountered include that of evaporation from the phosphate buffer during incubation. This has been overcome by using the lids of the tissue culture vessels, but the level of dissolved oxygen in the media may be affected. If hormone analysis indicates a fall off in the rate of steroid synthesis, this may be a result of the death of the follicle cells, which may be due to low oxygen levels.

It also appeared that there was a need for the randomisation of the position of chambers and the respective SPE concentration as there was a tendency for GV observation bias. A coding system, not known to the observer would overcome this problem, and this was adopted in subsequent experiments.

The oocytes appear to be robust and this will improve as the oocytes increase in size as more mature grayling are sampled. The counting of oocytes into each chamber was fairly consistent but some oocytes were damaged in handling and this must be improved.

2.2 Experiment 2: 31 January 1989

The results of Experiment 1 (December 1988) in this series of *in vitro* studies demonstrated that the grayling oocytes taken in early December could not be stimulated to complete final maturation and ovulation after treatment with SPE. It was clear from the small size of the oocytes that

Table 5 Somatic analyses taken from female grayling used in the in vitro experiment 2

Female	Length (cm)	Weight (g)	Ovary Weight (g) (BW%)	Mean Oocyte Diameter (mm)	G V Position (%)			
					2	3	3+	4
1	38.5	779	94 (12)	2.85	18	71	11	0
2	36.8	654	77 (12)	2.55	24	62	14	0
3	35.4	526	70 (13)	2.54	8	60	32	0

Table 6 Germinal vesicle position in grayling oocytes during in vitro incubation with salmon pituitary extract

Female 1

Time (hours)	8				16				24				48			
GV / SPE (ug ml^{-1})	2	3	3+	4	2	3	3+	4	2	3	3+	4	2	3	3+	4
0	4	94	2	0	9	81	11	0	19	76	5	0	17	65	19	0
0.01	14	79	7	0	19	72	9	0	18	73	9	0	22	72	6	0
0.1	7	86	7	0	12	80	8	0	24	68	7	2	12	73	16	0
1.0	6	87	7	0	12	78	10	0	19	75	7	0	24	65	11	0
10.0	15	65	20	0	25	66	8	0	10	80	10	0	13	76	11	0
100.0	9	88	3	0	8	78	13	0	15	77	8	0	15	68	17	0

Table 7 Germinal vesicle position in grayling oocytes during in vitro incubation with salmon pituitary extract

Female 2

Time (hours)	8				16				24				48			
GV / SPE (ug ml^{-1})	2	3	3+	4	2	3	3+	4	2	3	3+	4	2	3	3+	4
0	12	81	7	0	5	82	12	2	15	71	14	0	3	61	34	0
0.01	12	79	9	0	5	61	32	2	18	71	11	0	5	70	25	0
0.1	20	78	2	0	5	78	17	0	7	82	11	0	3	67	30	0
1.0	19	76	5	0	12	67	21	0	23	72	6	0	7	62	31	0
10.0	12	81	7	0	5	79	15	2	27	66	7	0	4	50	46	0
100.0	12	80	8	0	3	70	27	0	17	75	8	0	2	71	25	2

Table 8 Germinal vesicle position in grayling oocytes during in vitro incubation with salmon pituitary extract

Female 3

Time (hours)	8				16				24				48			
GV / SPE (ug ml^{-1})	2	3	3+	4	2	3	3+	4	2	3	3+	4	2	3	3+	4
0	6	82	12	0	4	79	17	0	7	61	30	2	4	44	52	0
0.01	9	80	11	0	2	61	35	2	4	89	7	0	0	53	47	0
0.1	13	68	19	0	0	57	40	3	4	76	18	2	2	58	38	2
1.0	3	75	14	8	0	66	32	2	8	70	17	5	0	51	49	0
10.0	9	77	14	0	11	70	19	0	7	54	39	0	6	62	32	0
100.0	4	83	13	0	16	66	18	0	5	73	20	2	2	67	31	0

vitellogenesis was still proceeding at this time. Further, the position of the GVs were between stages 2 and 3. Since the oocytes were small and no stimulation of GVM had occurred after SPE treatment, this led to the conclusion that germinal vesicle migration (GVM) had probably not been initiated *in vivo*.

The grayling used in this second *in vitro* experiment were taken some 8 weeks later than those used in Experiment 1. During this time, the grayling have been exposed to in excess of 400 degree days and have passed the minimum photoperiod at the winter solstice. Vitellogenesis should have been progressing and the initiation of GVM may also have occurred. As stated in Experiment 1, the likely effect of too high a gonadotropin dose is that of non-viability if the oocytes have commenced GVM. Similarly, a lower dose should promote GVM, initiate GVBD and effect ovulation.

Materials and Methods

Animals

5 female grayling were electrofished by personnel from Yorkshire NRA and Anglian NRA from the West Beck on 31 January 1989. The fish were transported to the Regional Fisheries Laboratory, Brampton for subsequent experimental use. Essentially, the methodology is the same as that used in Experiment 1.

Materials

A stock solution of salmonid pituitary extract (Argent, USA) of 1 mg ml^{-1} in phosphate buffered saline (pH 7.5) containing 10 g l^{-1} glucose, was prepared. Serial 10 fold dilutions of the stock in PBS were made to form stocks from 0.01 to 100 ug ml^{-1}. 2 ml of each of the five stock solutions were aliquotted out in duplicate into separate chambers, and 2 ml of PBS, in duplicate, was pipetted into the remaining two chambers. All treatments were randomised such that the concentrations were completely unknown to the observer.

Methods

Three female grayling were killed and after taking a blood sample for ovarian steroid analysis, somatic data were recorded. The ovaries were dissected out and groups of 30 oocytes were placed in each of the 12 chambers in identical tissue culture trays as used in Experiment 1. The trays were placed in a chest incubator, maintained at a temperature of 10 °C, four trays being prepared for each fish used in the experiment. A tray was taken for each fish, at 8, 16, 24 and 48 hours for analysis. During this analytical procedure, 1 ml of PBS was removed from each chamber and stored at -20

°C, and the remainder discarded. Clearing solution was added to the oocytes in each chamber and the position of the germinal vesicle noted after clearing. Oocytes were discarded after analysis. All observations were performed by one operator.

100 oocytes from each female were cleared in a clearing solution of 65 % glacial acetic acid in IMS, and the positions of the germinal vesicles classified. A mean oocyte diameter was also determined for each fish. These analyses were performed on stored oocytes after the incubation series to avoid prejudicing any experimental observations.

Results

Table 5 summarises the somatic analyses for the donor grayling, and also the positions of the GVs before incubation. Initial observations show that oocyte diameter has significantly increased in the intervening weeks and the positions of the GVs appears to be more eccentric, with more GVs at stage 3+. After incubation with SPE at all concentrations (Tables 6 to 8), no promotion of the GVs had occurred even after 48 hours. Disruption of the oocytes also occurred, but this was random. The PBS samples taken from the chambers during incubation were taken for ovarian steroid analysis to reveal the steroidogenic activity of the oocyte follicles after GtH stimulation.

Discussion

The results of Experiment 2 are similar to those of Experiment 1 in that little or no change occurred in the position of the GVs in each individual fish during incubation in a range of SPE concentrations. This leads to two conclusions: either the oocytes have not complete vitellogenesis and have therefore not commenced final maturation; or, if the oocytes have commenced final maturation, SPE is ineffective in stimulating final maturation and ovulation in vitro within the duration of the experiment. Given that CPE had catastrophic effects on the viability of oocytes in vivo as observed in March - April 1988, albeit at a later stage of development, as the grayling reach that same stage of development this year then some effects of the SPE should be observed. No induced spawning experiments were performed until early March 1988 and in this present study, these incubations were performed some five weeks in advance of similar stages of development. Thus, the next proposed experiment should take place in early March for a direct comparison. Similarly, an in vitro incubation with CPE should be performed as a direct comparison to determine whether species-specificity of the GtH is a cause of non-viability as well as the concentration of hormone.

The oocytes had developed further between early December and

late January. Clearly, oocyte diameter had increased and the GVs were more eccentric than in December. Steroid analyses should elucidate whether the production of maturation-mediating steroids was stimulated by SPE treatment and therefore determine whether vitellogenesis was completed. The next experiment, proposed for early March, will represent the first direct comparison with data obtained in 1988. Thus far, however, it is apparent that hormonal induction of maturation and ovulations cannot be performed *in vitro* in grayling some 8 weeks before natural spawning.

2.3 Experiment 3: 6th March 1989

The results of Experiment 2 (January 1989) in this series of *in vitro* studies demonstrated that the grayling oocytes taken at that time could not be stimulated to complete final maturation and ovulation after treatment with SPE. Oocyte growth had continued, resulting in a larger oocyte diameter than had been observed in December. Similarly, GV position had advanced. However, the absence of any promotion of GV position after SPE treatment suggests several possibilities. Firstly, GVM had not commenced *in vivo* and therefore the eccentric position was an artifact of the large oocyte size. Secondly, the hormone was not effective in stimulating GVM, either due to low biological activity, inappropriate concentrations or poor compatibility with the grayling oocytes, or further, although SPE was present, the addition of other hormones was necessary to effect GVM.

This later trial, some five weeks after the second experiment, was designed to address some of these hypotheses. Apart from the usual SPE treatments, a further set of treatments using carp pituitary extract (CPE) was also tested to elucidate any hormone specificity effects. In the research performed in 1988, the first *in vivo* experiment was performed in early March and this present *in vitro* trial was the first direct comparison between *in vivo* and *in vitro* treatments with artificially administered hormones at the same time during ovarian development. As stated in Experiments 1 and 2, the likely effect of too high a gonadotropin dose is that of non-viability if the oocytes have commenced GVM. Similarly, a lower dose should promote GVM, initiate GVBD and effect ovulation.

Materials and Methods

Animals

6 female grayling were electrofished by personnel from Yorkshire NRA and Anglian NRA from the West Beck on 6th March 1989. The fish were transported to the Regional Fisheries Laboratory, Brampton for subsequent experimental use. Essentially, the methodology is the same as that used in

Experiments 1 and 2.

Materials

A stock solution of salmonid pituitary extract (Argent, USA) of 1 mg ml^{-1} in phosphate buffered saline (pH 7.5) containing 10 g l^{-1} glucose, was prepared. Serial 10 fold dilutions of the stock in PBS were made to form stocks from 0.01 to 100 ug ml^{-1}. 2 ml of each of the stock solutions were aliquotted out in duplicate into separate chambers, and 2 ml of PBS, in duplicate, was pipetted into the remaining two chambers. All treatments were randomised such that the concentrations were unknown to the observer. A similar rationale was employed for the dilutions of CPE.

Methods

Three female grayling were killed and after taking a blood sample for ovarian steroid analysis, somatic data were recorded. The ovaries were dissected out and groups of 30 oocytes were placed in each of the 12 chambers in identical tissue culture trays was used in Experiment 1. Eight trays were prepared for each fish used in the experiment, four trays containing SPE and four CPE. The trays were placed in a chest incubator, maintained at a temperature of 10 °C. Two trays were taken at one of the four time intervals during the experiment, 8, 16, 24 and 48 hours for analysis, one tray from CPE and SPE treatments.

At each of the four time intervals, two trays from each female grayling were analysed, one for each hormone. 1 ml of PBS was removed from each chamber and stored at -20 °C, and the remainder discarded. Clearing solution was added to the oocytes in each chamber and the position of the germinal vesicle noted after clearing. Oocytes were discarded after analysis. All observations were performed by one operator.

100 oocytes from each female were cleared in a clearing solution of 65 % glacial acetic acid in IMS, and the positions of the germinal vesicles classified. A mean oocyte diameter was also determined for each fish. These analyses were performed on stored oocytes after the incubation series to avoid prejudicing any experimental observations.

Results

Table 9 summarises the somatic analyses for the donor grayling, and also the positions of the GVs before incubation. Initial observations show that oocyte diameter has increased in the intervening weeks but GV positions appear to be little changed. After incubation with CPE at all concentrations (Tables 10, 12 & 14), and SPE at all concentrations (Tables 11, 13 & 15) no promotion of the GVs had occurred even after 48 hours. Disruption of the oocytes

Table 9 Somatic analyses taken from female grayling used in the in vitro experiment 3

Female	Length (cm)	Weight (g)	Ovary Weight (g) (BW%)	Mean Oocyte Diameter (mm)	G V Position (%)			
					2	3	3+	4
1	31.5	387	55 (14)	2.63	10	52	31	7
2	33.0	432	43 (10)	2.67	0	58	28	14
3	30.2	335	36 (11)	2.87	0	64	36	0

Table 10 Germinal vesicle position in grayling oocytes during in vitro incubation with carp pituitary extract

Female 1

Time (hours)	8				16				24				48			
GV / CPE (ug ml^{-1})	2	3	3+	4	2	3	3+	4	2	3	3+	4	2	3	3+	4
0	10	52	31	7	0	58	42	0	0	61	39	0	0	49	51	0
0.01	7	75	13	5	0	58	42	0	0	64	36	0	0	50	50	0
0.1	2	79	19	0	0	46	54	0	0	51	49	0	0	58	42	0
1.0	0	84	14	2	0	46	54	0	0	60	40	0	0	40	60	0
10.0	5	72	20	3	0	52	48	0	0	43	57	0	0	50	50	0
100.0	0	65	35	0	0	51	49	0	0	52	78	0	0	40	60	0

Table 11 Germinal vesicle position in grayling oocytes during in vitro incubation with salmon pituitary extract

Female 1

Time (hours)	8				16				24				48*			
GV / SPE (ug ml^{-1})	2	3	3+	4	2	3	3+	4	2	3	3+	4	2	3	3+	4
0	0	39	56	5	0	53	42	5	0	49	51	0	non / viable			
0.01	0	54	42	4	0	70	30	0	0	61	39	0	non / viable			
0.1	0	58	43	0	0	59	41	0	0	56	44	0	non / viable			
1.0	0	47	52	1	0	64	36	0	0	70	30	0	non / viable			
10.0	0	41	56	3	0	40	57	3	0	61	39	0	non / viable			
100.0	0	58	42	0	0	41	54	5	0	65	35	0	non / viable			

* denotes that the oocytes were either disrupted or opaque on the addition of glacial acetic solution and that any readings would have been inaccurate

Table 12 Germinal vesicle position in grayling oocytes during in vitro incubation with carp pituitary extract

Female 2

Time (hours)	8				16				24				48*			
GV / CPE (ug ml^{-1})	2	3	3+	4	2	3	3+	4	2	3	3+	4	2	3	3+	4
0	0	58	28	14	0	70	30	0	0	60	40	0	non / viable			
0.01	0	57	29	14	0	67	33	0	0	62	38	0	non / viable			
0.1	3	78	16	3	0	67	33	0	0	67	33	0	non / viable			
1.0	0	55	35	10	0	59	41	0	0	63	37	0	non / viable			
10.0	0	65	30	5	0	52	48	0	0	64	36	0	non / viable			
100.0	0	58	38	4	0	61	39	0	0	78	22	0	non / viable			

* denotes that the oocytes were either disrupted or opaque on the addition of glacial acetic solution and that any readings would have been inaccurate

Table 13 Germinal vesicle position in grayling oocytes during in vitro incubation with salmon pituitary extract

Female 2

Time (hours)	8				16				24				48			
GV / SPE (ug ml^{-1})	2	3	3+	4	2	3	3+	4	2	3	3+	4	2	3	3+	4
0	0	60	40	0	0	55	45	0	0	55	41	4	0	49	51	0
0.01	2	55	43	0	0	62	38	0	0	57	39	4	0	39	61	0
0.1	0	51	47	2	0	54	46	0	0	55	45	0	0	60	37	3
1.0	0	62	37	1	0	52	48	0	0	74	26	0	0	53	47	0
10.0	0	53	45	2	0	59	41	0	0	55	43	0	0	50	50	0
100.0	0	59	41	0	0	68	32	0	0	59	41	0	0	50	50	0

Table 14 Germinal vesicle position in grayling oocytes during in vitro incubation with carp pituitary extract

Female 3

Time (hours)	8				24				48*			
GV / CPE (ug ml^{-1})	2	3	3+	4	2	3	3+	4	2	3	3+	4
0	0	64	36	0	0	49	51	0	non / viable			
0.01	4	67	30	0	0	53	47	0	non / viable			
0.1	0	57	43	0	0	63	37	0	non / viable			
1.0	0	60	40	0	0	64	36	0	non / viable			
10.0	0	51	49	0	0	67	33	0	non / viable			
100.0	0	68	32	0	0	67	33	0	non / viable			

* denotes that the oocytes were either disrupted or opaque on the addition of glacial acetic solution and that any readings would have been inaccurate

Table 15 Germinal vesicle position in grayling oocytes during in vitro incubation with salmon pituitary extract

Female 3

Time (hours)	8				16				24				48			
GV / SPE (ug ml^{-1})	2	3	3+	4	2	3	3+	4	2	3	3+	4	2	3	3+	4
0	0	47	47	6	0	71	29	0	0	53	47	0	0	38	62	0
0.01	0	52	48	0	0	66	32	2	0	59	41	0	0	57	43	0
0.1	0	56	40	4	0	60	40	0	0	65	35	0	0	43	57	0
1.0	2	63	34	1	0	59	41	0	0	55	45	0	0	37	63	0
10.0	0	61	39	0	0	66	34	0	0	53	47	0	0	31	69	0
100.0	1	54	43	2	0	62	38	0	0	49	51	0	0	44	56	0

also occurred, but this was generally random. Some groups of oocytes were completely disrupted on the addition of glacial acetic acid and in these the GV results have not been taken. The PBS samples taken from the chambers during incubation were taken for ovarian steroid analysis to reveal the steroidogenic activity of the oocyte follicles after GtH stimulation.

Discussion

The results from this experiment have shown again that GVM is not promoted during incubation with SPE at several concentrations. In this experiment also, the comparative study with corresponding amounts of CPE also failed to promote GVM. From the studies performed in 1988, it was postulated that at this stage of development either GVM, GVBD and ovulation or non-viability would occur *in vivo* after the administration of CPE, and/or SPE. This suggests that *in vitro*, the SPE / CPE treatment is failing to stimulate GVM due to one or more of several factors. Firstly, the hormone may not be in a high enough concentration, or be potent to achieve the desired effects. There may also be a missing "factor" between GtH stimulation of steroidogenesis and the effecting of GVM, GVBD or ovulation. Also, the hormone may require a longer time to complete these physiological changes than was available in this experiment. The potential phylogenetic differences between hormones have been shown not to be the major factors at this time. An *in vivo* study would now be the next appropriate experiment to give comparative data between *in vitro* (no promotion of GVM) and *in vivo* (GVM, GVBD, or non-viability).

The oocytes were larger in these fish than those taken for the second *in vitro* study. However, the fish used in that study were much larger than the donor fish in this experiment. A relationship has been elucidated between oocyte size and body length/weight in some fish species and this appears to be the case in grayling. As such, oocyte size may therefore be greatly influenced by body size, masking any comparative trends in oocyte growth and development. Therefore, the use of GSI may be a more useful indicator of oocyte development when taking into account the effects of body size and weight. In this case, GSI had changed little over the intervening period between this and the previous experiment and suggests that oocyte growth may have reached its completion by the previous sample taken in January.

Without histological data on oocyte structure and biochemical composition, however, the oocyte diameter measurements must be treated with caution as the body size of the fish varied between samples. The next experiment should test the effectiveness of SPE and CPE both *in vitro*, to compare with

all previous data, and in vivo, to determine whether the same results can be achieved as in 1988, or as those observed in vitro in 1989.

2.4 Experiment 4: 29th March 1989

This experiment was set up as the first comparative trial between in vitro and in vivo protocols. The aims were to repeat the SPE and CPE treatments in vitro as in previous experiments, to determine pre-spawning readiness by effecting GVM, GVBD and possibly ovulation. This was in conjunction with an in vivo trial to test the effectiveness of a range of hormone doses on gravid grayling in causing GVM, GVBD and ovulation.

Materials and Methods

Animals

33 female grayling were electrofished by personnel from Yorkshire NRA and Anglian NRA from the West Beck on 29th March 1989. The fish were transported to the Regional Fisheries Laboratory, Brampton for subsequent experimental use.

Materials

A stock solution of salmonid pituitary extract (Argent, USA) of 1 mg ml^{-1} in phosphate buffered saline (pH 7.5) containing 10 g l^{-1} glucose, was prepared. Serial 10 fold dilutions of the stock in PBS were made to form stocks from 0.1 to 100 ug ml^{-1}. 2 ml of each of the stock solutions were aliquotted out in duplicate into separate chambers, and 2 ml of PBS, in duplicate, was pipetted into the remaining two chambers. All treatments were randomised such that the concentrations were unknown to the observer. A similar rationale was employed for the single CPE treatment of 100 ug ml^{-1}.

Methods

Two female grayling were killed and a blood sample was taken for ovarian steroid analysis. The ovaries were dissected out and groups of 30 oocytes were placed in six chambers in identical tissue culture trays used in Experiment 1. Four trays were prepared for each fish used in the experiment, containing four SPE, one CPE and one control treatments. The trays were placed in a chest incubator, maintained at a temperature of 10 °C. A tray was taken at one of five time intervals during the experiment, 1, 8, 16, 24 and 48 hours for analysis. The remaining fish were used for in vivo studies.

At each of the time intervals, a tray from both female grayling analysed. 1 ml of PBS was removed from each chamber

and stored at -20 °C, and the remainder discarded. Clearing solution was added to the oocytes in each chamber apart from the 1 hour sample, and the position of the germinal vesicle noted after clearing. Oocytes were discarded after analysis. All observations were performed by one operator.

100 oocytes from each female were cleared in a clearing solution of 65 % glacial acetic acid in IMS, and the positions of the germinal vesicles classified. A mean oocyte diameter was also determined for each fish. These analyses were performed on stored oocytes after the incubation series to avoid prejudicing any experimental observations.

Results

Initial observations from Tables 16 and 17 show that GV positions appear to be little changed from experiment 3. After incubation with SPE and CPE, promotion of the GVs had not occurred even after 48 hours. The PBS samples taken from the chambers during incubation were taken for ovarian steroid analysis to reveal the steroidogenic activity of the oocyte follicles after GtH stimulation.

Discussion

The results from this experiment have shown again that GVM is not promoted during incubation with SPE at several concentrations and CPE at 100 ug ml^{-1}. The *in vivo* experiment should elucidate whether the oocytes are indeed sufficiently mature and therefore in a receptive condition to hormonal treatment. This should indicate the likely reasons for the lack of positive results in the *in vitro* experiments.

3 EXPERIMENTS TO INDUCE OVULATIONS IN GRAVID FEMALE GRAYLING

3.1 *in vivo* trials with SPE and CPE

The results obtained from the series of *in vitro* experiments, designed to predict pre-spawning readiness, were not as expected. No confirmational changes had occurred after hormonal stimulation and little information was determined initially. Data on steroid production was to be retrospective and was not available to aid the spawning programme. Hence, a trial experiment was designed to determine whether the administration of hormone to the fish *in vivo* caused any of the observed structural changes observed in 1988, or the desired changes of promotion of GV position GVBD and ovulation. The hormones to be tested were CPE, containing carp GtH, which was used in 1988 and SPE, the GtH contained within this preparation being phylogenetically closer to grayling GtH than the carp GtH. For the comparative aspects between the 1988 and 1989 trials, similar hormone levels of both hormones were tested.

Table 16 Germinal vesicle position in grayling oocytes during in vitro incubation with salmon and carp pituitary extract

Female 1

Time (hours)		8				16				24				48			
	GV	2	3	3+	4	2	3	3+	4	2	3	3+	4	2	3	3+	4
SPE ($\mu g\ ml^{-1}$)	0	0	64	36	0	0	60	40	0	0	25	75	0	0	70	30	0
	0.1	0	50	50	0	0	48	52	0	0	69	31	4	0	66	34	0
	1.0	0	46	54	0	0	79	21	0	0	79	21	0	0	76	24	0
	10.0	0	41	59	0	0	67	33	0	0	67	33	0	0	65	35	0
	100.0	0	48	52	0	0	50	50	0	0	86	15	0	0	67	33	0
CPE	100.0	0	73	27	0	0	64	36	0	0	64	36	0	0	68	32	0

Table 17 Germinal vesicle position in grayling oocytes during in vitro incubation with salmon and carp pituitary extract

Female 2

Time (hours)	8				16				24				48			
GV	2	3	3+	4	2	3	3+	4	2	3	3+	4	2	3	3+	4
SPE (ug ml^{-1}) 0	0	26	61	13	0	48	52	0	0	21	79	0	0	33	67	0
0.1	0	20	76	4	0	4	92	4	0	33	67	0	0	23	77	0
1.0	0	42	50	8	0	15	85	0	0	41	59	0	0	33	67	0
10.0	0	23	73	4	0	19	81	0	0	44	56	0	0	5	95	0
100.0	0	12	76	12	0	17	69	14	0	25	71	4	0	28	72	0
CPE 100.0	0	23	69	8	0	48	48	4	0	40	60	0	0	4	96	0

A 1 hour incubation was performed for the steroid sample, but no GV analyses were taken.

Materials and Methods

Animals

33 female grayling were electrofished by personnel from Yorkshire NRA and Anglian NRA from the West Beck on 29th March 1989. The fish were transported to the Regional Fisheries Laboratory, Brampton for subsequent experimental use. Two fish were used for the *in vitro* study (2. 4) and the remainder for these experiments.

Materials

Salmonid pituitary extract and carp pituitary extract (Argent USA) were diluted serially from 10 mg ml^{-1} to 0.02 mg ml^{-1} to form the appropriate concentrations from 5 mg kg^{-1} to 0.01 mg kg^{-1} for *in vivo* treatment.

Methods

Groups of female grayling were segregated into cages within two 3,000 l tanks, both containing water at approximately 10 °C, and each group was given a hormone treatment by intraperitoneal injection, as shown in Table 18. After 72 hours, individuals from each group were biopsied to obtain gonad material. Oocyte structure and maturity (GV position) was noted after clearing and further hormone treatments were given in certain groups. No biopsied fish were given further hormonal treatments but their physiological condition was noted at each examination. Fish were inspected daily for changes in secondary sexual characteristics and signs of ovulation. Ovulating females were handstripped and eggs fertilised using standard *in vitro* methodologies. Oocyte development was followed during incubation. Malachite green treatments were administered each time the fish were handled.

Results

Tables 19 and 20 show the results of the *in vivo* experiment. The biopsy samples taken three days after the first hormonal treatment revealed differences in the oocyte structure between groups. Oocytes taken from females of groups 1 and 2 were completing the final maturation processes. These oocytes had gone beyond GVBD and yolk agglutination stages but were not ovulated. Oocytes from females of group 3 were more mature than the control fish, but GVBD had only occurred in a few oocytes in one individual. Slight development in secondary sexual characteristics had occurred. Analysis of oocytes from female grayling in group 4 revealed that there was little change after the first hormonal injection. The second injection, administered on day 3, caused some confirmational changes by day 6. Neither hormonal treatments administered to grayling of group 5 caused changes to oocyte maturity or structure. No changes occurred to the oocytes

Table 18 Hormonal treatment regimes used on gravid female grayling from the West Beck

Group No.	n	treatment	date of treatment	n biopsied 3.4.89
1	3	5 mg kg^{-1} CPE,	31.3.89	3
2	3	5 mg kg^{-1} SPE,	31.3.89	3
3	3	1 mg kg^{-1} SPE,	31.3.89	3
4	7	0.1 mg kg^{-1} SPE, 1.0 mg kg^{-1} SPE,	31.3.89 3.4.89	3
5	11	0.01 mg kg^{-1} SPE 0.1 mg kg^{-1} SPE 1.0 mg kg^{-1} SPE	31.3.89 3.4.89 5.4.89	3 3
6	4	0.5 ml kg^{-1} saline	31.3.89	2

Table 19 Results after hormonal treatment in gravid female grayling

Group No.	n	treatment	oocyte structure 3.4.89
1	3	5 mg kg^{-1} CPE	All oocytes flaccid. Aggregated yolk globules of regular shape and size. Beyond GVBD. Oocytes quickly burst in clearing solution. Larger, red coloured genital papillae.
2	3	5 mg kg^{-1} SPE	Oocytes less flaccid than those from group 1. Aggregated yolk globules of regular shape. Beyond GVBD. Oocytes slowly burst in clearing solution Larger, red coloured papillae.
3	3	1 mg kg^{-1} SPE	Yolk evenly distributed in oocytes, slight agglutination. GVs 2 3 3+ 4 GVBD 0 0 17 79 4 0 0 40 60 0 0 0 10 90 0 Oocytes crinkled in clearing solution Papillae not as developed as gps 1 & 2
4	3	0.1 mg kg^{-1} SPE	Oocytes have usual pre-spawning structure. Little agglutination of yolk or crinkling of chorion in clearing solution. Little change in papilla appearence. GVs 2 3 3+ 4 0 0 78 22 0 11 78 11
	4	1.0 mg kg^{-1} SPE	(6.4.89) More agglutination and GVBD in some oocytes. Large improvement in papilla appearence.
5	3	0.01 mg kg^{-1} SPE	Condition as with group 4 fish. GVs 2 3 3+ 4 0 0 75 25 0 0 79 21 0 8 92 0
	4	0.1 mg kg^{-1} SPE	(6.4.89) Little change from 3.4.89 biopsy sample.
	4	1.0 mg kg^{-1} SPE	Some improvement to oocyte structure
6	4	0.5 ml kg^{-1} saline	No agglutination of yolk in oocytes GVs 2 3 3+ 4 0 35 65 0 0 44 56 0

Table 20 Results after hormonal treatment in gravid female grayling

Group No.	n	treatment	No. ovulations
1	3	5 mg kg^{-1} CPE	3, day 5 (5.4.89)
2	3	5 mg kg^{-1} SPE	3, day 5 (5.4.89)
3	3	1 mg kg^{-1} SPE	0, (7.4.89)
4	3	0.1 mg kg^{-1} SPE	0, (7.4.89)
	4	0.1 mg kg^{-1} SPE (31.3.89) 1.0 mg kg^{-1} SPE (3.4.89)	0, 0 (7.4.89)
5	3	0.01 mg kg^{-1} SPE	0, (7.4.89)
	4	0.01 mg kg^{-1} SPE (31.3.89) 0.1 mg kg^{-1} SPE (3.4.89	0, 0, (7.4.89)
	4	0.01 mg kg^{-1} SPE (31.3.89) 0.1 mg kg^{-1} SPE (3.4.89) 1.0 mg kg^{-1} SPE (5.4.89)	0, 0, 0, (7.4.89)
6	4	0.5 ml kg^{-1}	0, (7.4.89)

Experiment stopped on 7.4.89

from group 6 (control) females.

On days 4 and 5 of the experiment, further observations were made on all groups. Full ovulations were achieved in all fish from groups 1 and 2. No changes to oocyte structure or ovulations occurred in female grayling from groups 3 to 6 during this time. The experiment was stopped on day 7 when it was apparent that no further developments were likely to occur.

Discussion

There are several aspects to the results of the *in vivo* trial. Firstly, a single SPE or CPE hormonal treatment of 5 mg kg^{-1} was effective in completing GVM, initiating and completing GVBD and inducing ovulation. The time scale was also elucidated, GVM, GVBD and yolk agglutination having occurred by 72 hours after treatment. Ovulation took a further 48 hours to be initiated. Both CPE and SPE had these effects, which contrasts to those observed in 1988. In that series of experiments, CPE caused catastrophic effects to the oocyte structure. The present results suggest that the hormone used in the 1988 experiments was of inferior quality or caused other "side-effects", observed as oocyte "non-viability". However, as described and discussed in section 3.3, the oocytes ovulated after CPE, but not SPE treatment, appeared to be infertile, due to the absence of any cellular development after fertilisation. Secondary sexual characteristics developed after hormonal treatment in both groups compared to the controls, with the genital papilla becoming much larger and taking a deep red colour. The anal fin also thickened, which may be an adaptation to redd digging. The use of a high dose of SPE was effective in inducing ovulations of viable eggs and the initial viability of the developing embryos, the fertilisation rate, was high all three fish. Subsequent deterioration in embryo viability occurred 4 days after fertilisation. These aspects are discussed in section 3.3.

The low hormonal treatment of 1 mg kg^{-1} was effective in completing GVM in most oocytes and initiating some yolk agglutination. The secondary sexual characteristics of improved genital papilla shape and colour were not as advanced as in groups 1 and 2 but were advanced over those of the control fish. Thus there is a dose related difference in the response to SPE treatment: the lower dose causes GVM to be completed, but a higher dose causes GVM, GVBD and ovulation.

In group 4 fish, little changes in the oocyte structure were detected after the first hormonal treatment, of 0.1 mg kg^{-1}. The second treatment, 1.0 mg kg^{-1} caused some confirmational changes to oocyte structure but and a great development in genital papilla condition was detected. Thus, after a low

SPE treatment of 0.1 mg kg^{-1}, no change to the stage of oocyte maturation was detected and an improvement in the state of maturation occurred only after a further treatment of a higher dose. Similar results were achieved in females from group 5, in which the first two hormonal treatments were ineffective in causing any development of oocyte maturation or secondary sexual characteristics.

This reveals two important factors. Primarily, there is a dose relationship between a single SPE injection of either 0.01 or 0.1 mg kg^{-1}, where no changes occurred up to 3 days after treatment and of 1.0 mg kg^{-1} (group 3) which effected GVM. Secondly, treatment with a gradual increase in hormonal levels, which should be close to the natural endogenous GtH profiles, was not more effective in completing GVM, GVBD and ovulation than the single SPE dose of 1.0 mg kg^{-1}.

In conclusion, this part of the experiment revealed that a single dose of 1.0 mg kg^{-1} SPE was effective in effecting GVM and a single dose of 5.0 mg kg^{-1} SPE caused GVM, GVBD and ovulation. Doses of 0.1 mg kg^{-1} or less were not effective in causing any detectable changes to oocyte structure relating to maturation or viability. CPE at 5 mg kg^{-1} effected GVM, GVBD and ovulation but subsequent embryo survival was nil.

3.2 Application of hormonal treatment to captive and wild pre-spawning female grayling

A protocol for the induction of ovulations was developed after the results obtained from the *in vivo* experiment (section 3.1). A dose regime should reflect as closely as possible the natural hormone profiles of the recipient fish. In salmonids, the GtH profile gradually increases in the pre-spawning period (Crim, Evans and Vickery, 1983). From this, a double dose regime would be preferred, using the information of the SPE treatment effects from the experiment, with a higher dose in the second injection. A 1 mg kg^{-1} SPE treatment caused the completion of final maturation in grayling oocytes. A higher dose of 5 mg kg^{-1} effected GVBD and ovulation at 10 °C but the threshold for the differences in effects between the two doses was probably 2 to 3 mg kg^{-1}. Thus the proposed regime was a priming dose of 1 mg kg^{-1} to complete GVM, and after 48 hours a second injection of 3 mg kg^{-1} to effect GVBD and ovulation. The time interval should be sufficient for the oocytes to complete GVM, and generally after this time, most GtH would have been either metabolised or excreted. Ovulation would be expected to occur after a further three to four days, but egg viability may last up to seven days after ovulation within the brood female.

The following hormonal treatment was used on a variety of grayling from various sources and oocyte development stages. Each group is treated and discussed separately, and Table 21 summarises the production for this year.

3.2.1 West Beck females

The West Beck female grayling which did not ovulate after the first in vivo experiment (groups 3 - 6) were given the hormonal treatment outlined, of 1 mg kg^{-1} at 0 hours and 3 mg kg^{-1} at 48 hours. The time to ovulation was variable in this group, probably reflecting the different hormonal treatments which the fish had in the previous week. Generally, females from group 3 and 4 ovulated before those of groups 5 and 6. Similarly, full ovulations were seldom obtained by day 5 and a given female had to be stripped over a further two to three days. Viability did not appear to be affected over this time although it was preferable to remove all ovulated eggs at each stripping. This avoided the continual problem of mixing up batches of eggs from different females and reduced stress to broodstock. Clearly, if all female grayling were left for stripping on day 6 instead of day 5, then full egg ovulations would be encountered.

3.2.2 Captive West Beck and R. Kennet females from 1988

The captive grayling from the West Beck and R. Kennet were maintained throughout 1988 and early 1989 in recirculation systems at Brampton. During that time, they were fed on a diet of maggots and pellets, although pelletted food was generally unpalatable. The condition of the female grayling was very good in the late summer and autumn of 1988, indicating that gonad material was being laid down. In the spring months of January and February, regular inspection were made of females and males to determine any secondary sexual development or pre-spawning readiness. In due course, male grayling commenced spermiation in January, some four weeks before the males in the West Beck. They also darkened and occasional pursuit swimming was observed in February. Little further development was discernible and no natural spawning occurred in the tank systems. In late-March a female grayling was biopsied to determine the stage of oocyte development. The oocyte was at the end of maturation, but they were also apigmented. This was of great significance as it was clear that the diet had been difficient in carotenoid pigments and accordingly a study was initiated to determine whether these oocytes were still viable and whether they could be ovulated.

A single female was given an SPE treatment of 5 mg kg^{-1} on 31st March. On 4th April, a full ovulation was achieved and development was followed during incubation. As already stated, embryo viability was low in all treatment groups, regardless of the history of the fish. This was also the

Table 21 Summary of grayling egg and fry production 1989

Total number of females spawned	=	> 35
Total number of eggs produced	=	~ 135,000
Total number of viable eggs produced	=	~ 125,000
Total number of larvae hatched	=	~ 20,000
Total number of larvae to first feeding	=	~ 15,000
Total number of larvae stocked out to Snailwell	=	1,120
Total number of fry produced	=	< 50

Losses through rearing due to bacterial infection (did not respond to antibiotics).

case for this captive fish, but development did continue to hatching. Initial post-hatching survival was very good, but a bacterial infection caused a great mortality in all alevins. Further hormonal treatments were applied to the 1988 West Beck fish and the survival was compared with that of the initial alevins produced. These results imply that the oocytes were indeed viable and embryo development was normal, despite the absence of pigment. However, the poor survival suggests that the alevins were more susceptible to damage, infection, or other environmental pressures at the alevin stage than pigmented larvae.

3.2.3 Captive R. Kennet females from 1989

In February of 1989, 80 grayling of mixed sex were obtained from the R. Kennet and introduced into recirculation systems. After several days, most fish were affected by bacterial infections, particularly Aeromonas and secondary infections of Saprolegnia. Treatment with an IP injection of Terramycin and regular treatments of Malachite green @ 0.1 ppm ensured that the majority of the fish survived. However, reproductive function is often affected by the stress of transport and handling and antibiotic treatments can cause regression of oocytes. Thus, these fish were not ideal broodstock and were not taken for spawning in the early part of the experimental period. In late April, when it was clear that more eggs were required, several females were taken for induced spawning. This was after the initial in vivo trials and therefore the induced spawning protocol was developed. In the first batch, four out of six females ovulated, whereas in the second some seven days later, 2 out of four females ovulated. Embryo development was as before, with a high mortality between days 3 to 6.

3.3 Male grayling

The problems encountered with handstripping male grayling have been documented in Brighty and Jordan (1988), but a brief summary is necessary for the results of this years work. Spermiation appears to be improved by treatment with SPE at 1 to 5 mg kg^{-1}. Viability on the first stripping was very high, but subsequent viability had to be checked routinely and activity was generally inconsistent after the first stripping. Tank maintained males appeared to darken by the end of January, with the dorsal and pectoral fins became irridescent blue and semen could be expressed from the vent by mid-February. The male secondary sexual characteristics were sufficiently distinctive to aid sexing, the dorsal fin reaching back to the adipose fin.

3.4 Experiments to determine causes of mortality during incubation

The standard fertilisation method was followed for the spawning of the majority of captive and wild grayling. After ovulation, each fish was stripped using the usual Woynarovich solution to enhance the fertilisation rate. After fertilisation, the eggs were rinsed with Woynarovich solution and placed in the trough systems at 8 to 11 °C. The pumps were not restarted until the eggs had fully hardened. Initial fertilisation rates appeared high but after a period of three to seven days, a great mortality was observed in all groups. This manifested itself as a sudden change in colour in the egg yolk, from yellow to white. This is caused by a rupturing of the vitellus, which allows sodium chloride to be lost from the yolk, thus precipitating out the yolk. The causes of such mortality was not clear. It was possible that the eggs were infertile, shown as an absence of cellular differentiation during development. This was the case for the grayling treated with CPE where no organised cellular development occurred. Thus, after a short period of time, the egg membrane begins to breakdown and through the loss of salt, precipitation occurs. The eggs could have received physical damage during the fertilisation process and until hardening, the vitelline membrane may be damaged by movement. More likely in most groups was that the water quality either during hardening and/or incubation was causing mortality. In particular, it could be through a direct route by the toxic effect of an unknown dissolved substance, low oxygen or high temperature. Alternatively, it may be indirect, such as through the presence of calcium salts in the water which may slow the extension rate of the chorion or even prevent the chorion from swelling to its potential size. Any of these effects could be compounded by the incubation methodology.

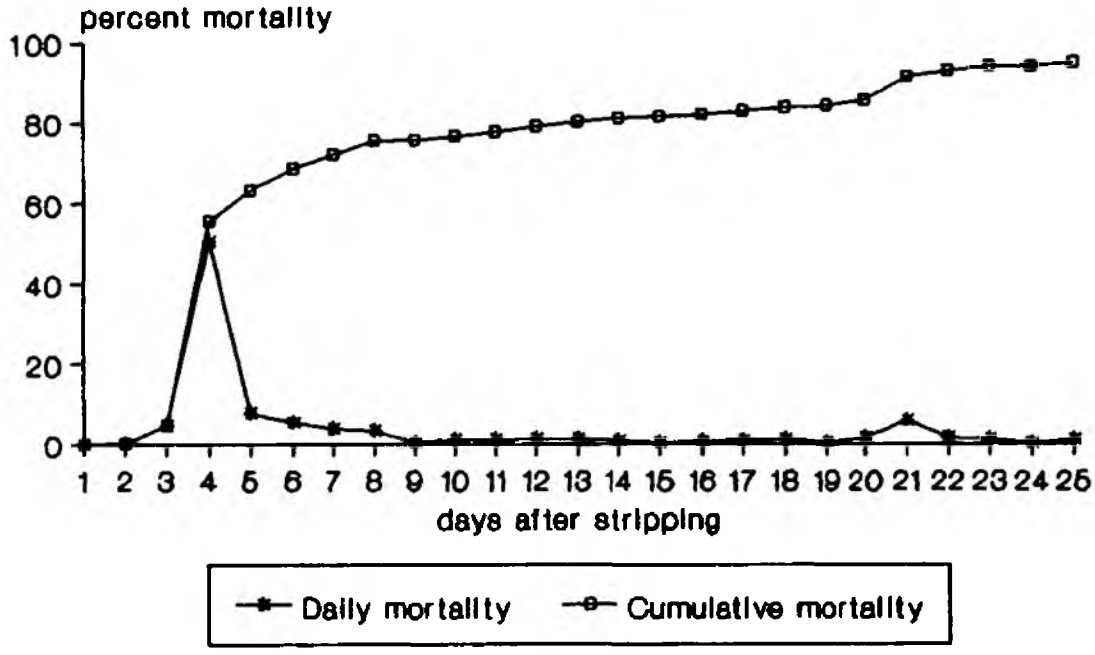
In order to determine the time of greatest mortality, mortality was plotted against time, both in absolute and cumulative terms (Figure 1) revealing that day 4 to 6 was the "critical" period. It was not clear what was causing the observed mortality, particularly whether the eggs were predisposed to poor survival due to some previous event, such as at fertilisation, or whether it was a function of incubation, for example water quality, disease or methodology. With this in mind, some experiments were set up to determine the main reasons for this mortality.

These were:

1. the effect of the fertilisation solution - tested Woynarovich solution vs a French fertilisation solution.

2. the effects of the hardening and incubation water and mode of incubation on size and survival of grayling embryos

Figure 1 Pattern of mortality in eggs stripped from a female grayling on 5 April 1989



3. hardening grayling eggs in a range of calcium solutions

Other precautions were taken to prevent the penetration of UV light into the troughs and wide fluctuations in temperature.

3.4.1 Fertilisation solutions

Eggs from the same donor female were split into two groups and each was fertilised using a fertilisation solution, one using Woynarovich, the other using a recipe from a French grayling research group (pers.comm.). The eggs were then placed in the same tray in an incubation trough. Mortality of eggs was followed over a 10 day period. The mortality was as recorded before in previous inductions of spawning, but no differences in observed mortality was recorded between groups. Hence, Woynarovich fertilisation solution was discounted as being the sole cause of mortality in the grayling eggs.

3.4.2 Hardening and incubation water chemistry and mode of incubation

A recent publication (Ketola et al., 1988) has demonstrated that high calcium levels in incubation water have a detrimental effect on the survival of trout and salmon eggs, and that the critical period in which this adverse effect takes place was during the so-called "hardening" stage. Hardening involves two concomitant processes and lasts for approximately one hour immediately after eggs are released into the water. One process is the uptake of water through the chorion resulting in a considerable increase in egg volume, while the other process involves the incorporation of calcium into the chorion. This imparts some rigidity into this membrane and further extension of the chorion is not possible after the membrane has fully hardened. Ketola et al (1988) demonstrated that eggs could be hardened in low-calcium water and then transferred to high calcium water for the incubation without adverse effect and that this method greatly increased survival over eggs hardened in high-calcium water.

An experiment was designed to assess the effect of the chemistry of hardening water on grayling egg incubation mortality. One female was stripped and all eggs were fertilised immediately in Woynarovich solution for 3 minutes. The eggs were split into two batches. One batch was hardened in 2l distilled water for one hour at 10 °C, then three subsamples were taken for incubation. Subsample 1 (205 eggs) was placed in 1l distilled water (D to D) in an incubator at 10 °C, subsample 2 (185 eggs) was placed in 1l borehole water (D to B) in the same incubator and the third subsample (830 eggs) was placed in a tray in the trough incubating system (AB(1)). The second batch of eggs was hardened in 2l borehole water for one hour at 10 °C and then also split into

three subsamples. Subsample 1 (169 eggs) was placed in 1l distilled water (B to D) in the incubator, subsample 2 (128 eggs) was placed in 1l borehole water (B to B) in the incubator and subsample 3 (3551 eggs) was placed in the same tray in the incubation system as AB1, but separated by a partition (AB(2)). The matrix is summarised in Table 22. Subsamples in the incubator were held in flat-bottomed glass dishes and were not aerated. Dead eggs were counted and removed once daily throughout the development period.

Several noteworthy trends were observed during the experiment. Up to 3 days after fertilisation, the mortality patterns were low and similar in all treatments (Figure 2), but between days 4 to 7, there was an increase in observed mortality in all groups hardened in borehole water, and both subsamples incubated in the trough system. This mortality followed a similar pattern to that observed in other previous incubations during earlier spawning trials. The two subsamples of eggs hardened in distilled water and incubated in distilled and borehole water had very low mortality (D to D, D to B). These trends continued for the remainder of the experiment but the final mortality level in each group, and the rate at which it occurred were not as anticipated (Figure 3). The lowest mortality was exhibited in the groups hardened in distilled water, and incubated in either distilled or borehole water. The duplicate treatment of hardening in distilled water and incubating in borehole in the trough system (AB 1) exhibited the greatest mortality in the eggs. This indicates that there is an adverse effect on the eggs by the trough incubation method, after hardening in distilled water. A similar effect was also observed in subsample AB 2, suggesting that the incubation methodology was indeed the major cause of observed mortality. However, water chemistry aspects may also have been of importance. Mortality in the B to B subsample was very high, at 84 % but eggs from the B to D group exhibited only 50 % mortality. Mortality in the subsample D to B during the latter period of the incubation period was an artifact, and embryo death was caused by water fouling.

This experiment revealed some important findings relating to the incubation of grayling eggs. Groups of eggs hardened in borehole water exhibited a minimum of 50 % mortality, and if also incubated in borehole water, the mortality increased to 84 %. If incubated in the trough system in borehole water, mortality was 95 %. When distilled water was used to harden the eggs, mortality was only 10 % when incubated in distilled water or 26 % if incubated in borehole water. However, when distilled water-hardened eggs were incubated in the trough system, mortality increased to 93 %.

Therefore, both water chemistry and the incubation method had great bearings on the ultimate viability of the embryos. Borehole water is unsuitable for hardening the grayling eggs,

Table 22 Matrix for water hardening experiment

D to D:	Distilled-water-hardened for 60 minutes and incubated for 16 days in distilled water in a chest incubator at 10 °C
D to B:	Distilled-water-hardened for 60 minutes and incubated for 16 days in borehole water in a chest incubator at 10 °C
B to D:	Borehole-water-hardened for 60 minutes and incubated for 16 days in distilled water in a chest incubator at 10 °C
B to B:	Borehole-water-hardened for 60 minutes and incubated for 16 days in borehole water in a chest incubator at 10 °C
AB(1)	Distilled-water-hardened for 60 minutes and incubated for 16 days in borehole water in a trough system at 10 °C
AB(2)	Borehole-water-hardened for 60 minutes and incubated for 16 days in borehole water in a trough system at 10 °C

Figure 2 Daily mortality of grayling eggs hardened and incubated in different water treatments

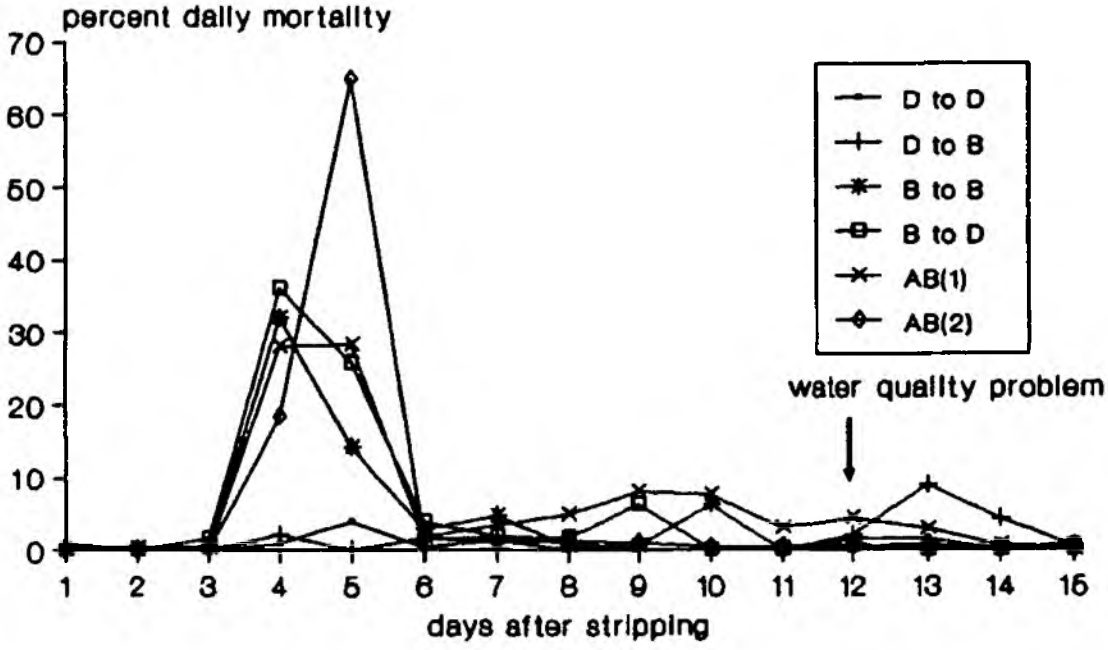
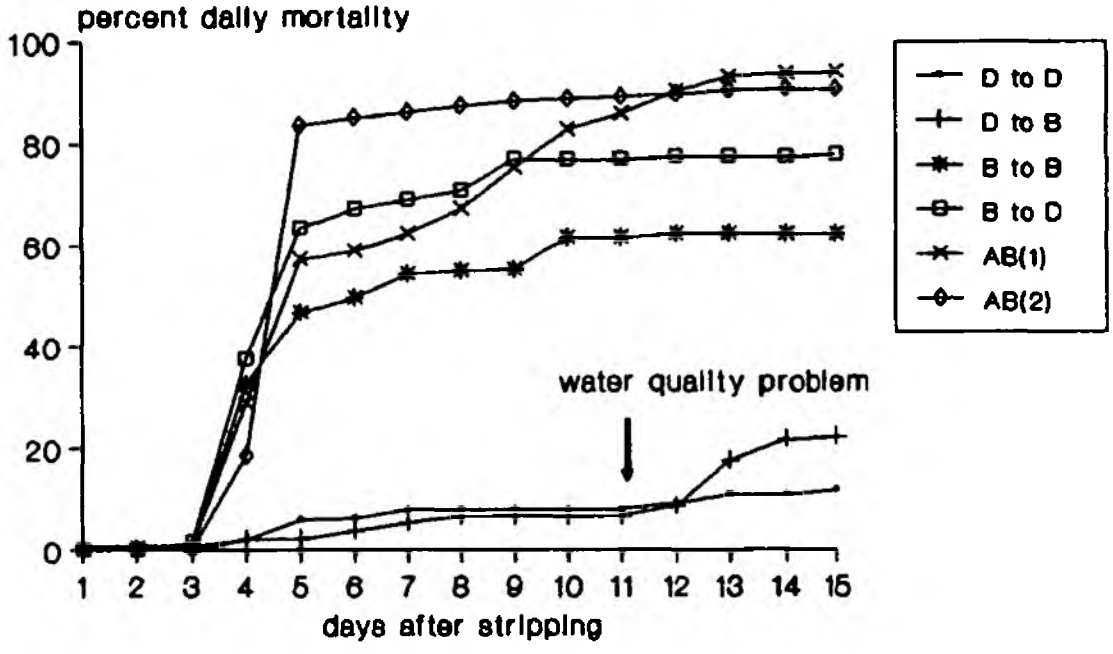


Figure 3 Cumulative mortality of grayling eggs hardened and incubated in different water treatments



resulting in greater than 50 % mortality. Incubation of eggs in borehole water results in significant mortality, particularly if also hardened in the same water. Various dissolved chemicals may be responsible for the mortality but these are as yet unknown. However, the total calcium level is high, at 200 mg l^{-1} and this reflected in the small ultimate size of the chorion in borehole hardened eggs, restricting the developing embryo. The effect of hardening water (distilled and borehole) on the swelling rate and ultimate egg size was determined in a separate experiment (Figure 4). Ultimate size was reached after 60 minutes and was significantly greater than that in borehole water, the final egg volume being 30.8 mm^3 and 21.4 mm^3 in distilled and borehole water water respectively. Transfer of a subsample of distilled-water-hardened eggs to borehole water after 200 minutes, and vice versa, did not result in a change in egg diameter, indicating that egg diameter is determined and fixed within 60 minutes. This is probably a reflection of the incorporation of calcium in the chorion which prevents further uptake even if the eggs are then transferred to water of lower ionic strength.

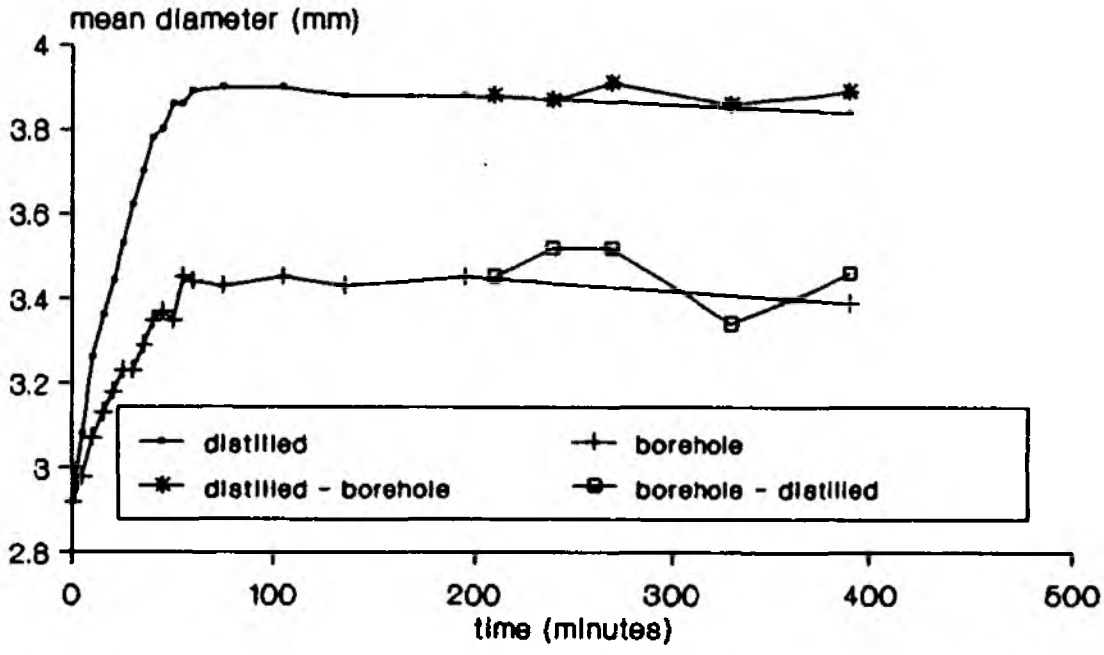
Hardening in distilled water reduces the mortality to around 10 %, but incubation in trough systems causes complete mortality. This may be because the chorion had not hardened sufficiently to protect the delicate embryo within during incubation. Certainly, the fastest mortality rate was observed in this group, implying a rapid mortality consistent with poor embryo protection, either from physical or chemical disturbances. Additions of calcium to distilled water for the hardening process may achieve the desired calcification and protection. For practical reasons, static incubation of the eggs is not satisfactory for large-scale production and alternative incubation methods must be found. The use of an alternative water supply might improve embryo survival.

3.4.3 The effect of calcium ions on the elevation of the chorion of fertilised eggs during water hardening and their survival during incubation

In experiment B, one of the observed effects was that over 80 % of the eggs hardened in distilled water and incubated in borehole water in the trough system were dead five days after fertilisation. This catastrophic mortality contrasted to the "replicate" treatment in the static chest incubator where mortality was less than 10 %. This implies that the quality of the borehole water did not directly cause the mortality of the eggs. However, the combined effects of distilled hardening water and borehole incubation water with the trough incubation method may have lead to the sudden demise of the developing embryos.

Calcium ions are important in the hardening process of the eggs, being incorporated into the chorion during its

Figure 4 Elevation of the chorion of grayling eggs after immersion in distilled or borehole water



elevation. It also adds to the osmotic potential of the surrounding water, which will regulate the rate at which water enters through the chorion into the perivitelline space around the grayling zygote. As the osmotic potentials equilibrate between the swelling egg and the external water, the chorion completes its hardening process and little further increases or decreases in egg volume are possible. Distilled water contains little dissolved calcium and thus there may be insufficient calcium to harden the egg completely. This would render the embryo unprotected from physical or chemical shocks. As distilled water was used to harden the eggs in preference to borehole water, due to the hypothesis that the borehole water quality was suspect, the absence of calcium may have lead to incomplete hardening, redisposing the developing embryo to endure physical and chemical disturbances.

This experiment was designed to test the effects of hardening grayling eggs in distilled water containing known concentrations of calcium chloride, on survival of developing embryos.

Grayling eggs were placed in hardening solutions of a range of concentrations of calcium chloride in distilled water, from 0 to 200 mg l^{-1}, and a further treatment group of borehole water. Egg diameter measurements were taken from all groups after the hardening process had been completed, approximately 1 hour after immersion in water. Each group of eggs was placed in glass bowls containing distilled water in the chest incubator. The incubator was set at 10 °C and the pattern of mortality was determined for all groups during incubation.

Mortality of grayling eggs did not appear to be dependent on the hardening solutions (Figures 5 and 6). The typical pattern of mortality observed in previous experiments, that of a commencement of yolk precipitation inside the vitelline membrane and perivitelline space by day 4 post fertilisation, occurred in all groups at similar rates. There were no observed differences between treatments with respect to mortality during early incubation. By day 8, over 80 % of the eggs had died.

The experiment is inconclusive in terms of testing the effects of different hardening solutions on survival. The trial which studied the effects of hardening water quality on initial survival revealed that hardening in distilled water and incubating in either distilled or borehole water in an incubator caused the least mortality during early incubation, at less than 10 %. This treatment was effectively repeated in this experiment, except that the eggs were from a different female. In view of this fact, and that all treatments exhibited similar mortality patterns, it is likely that the viability of the eggs used in this experiment was

Figure 5 Daily mortality of grayling eggs hardened in distilled water containing dissolved calcium

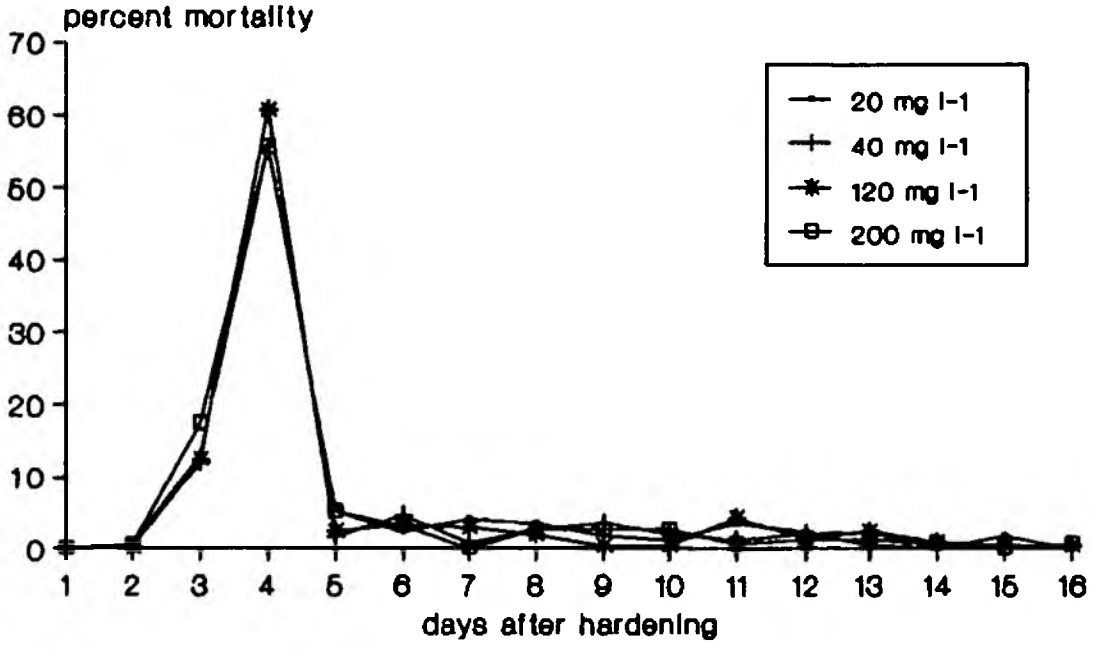
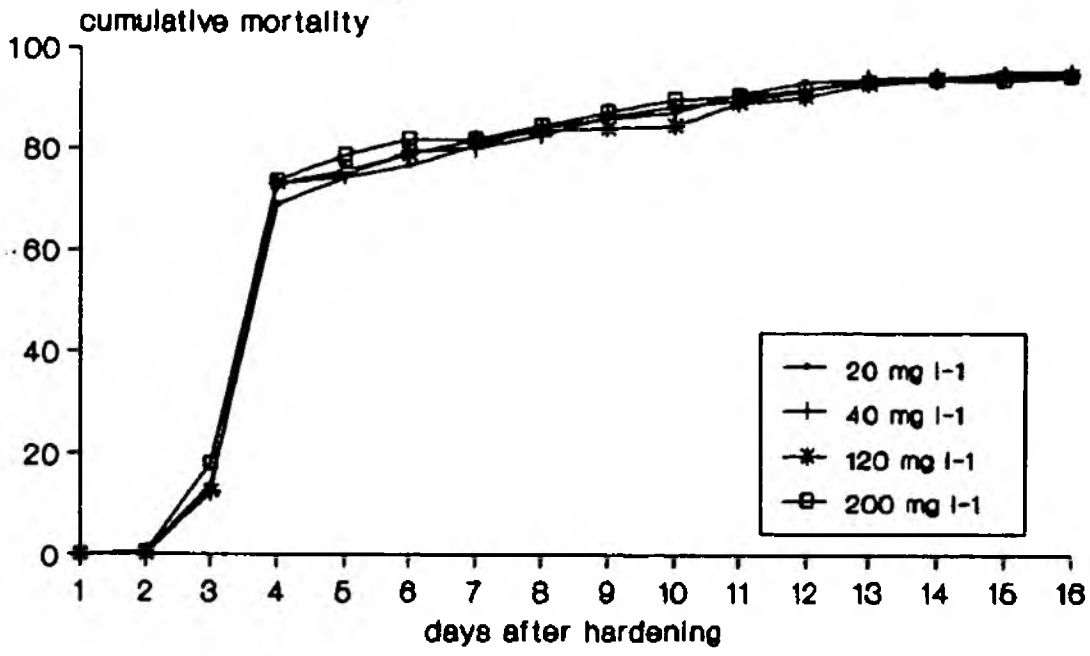


Figure 6 Cumulative mortality of grayling eggs hardened in distilled water containing dissolved calcium



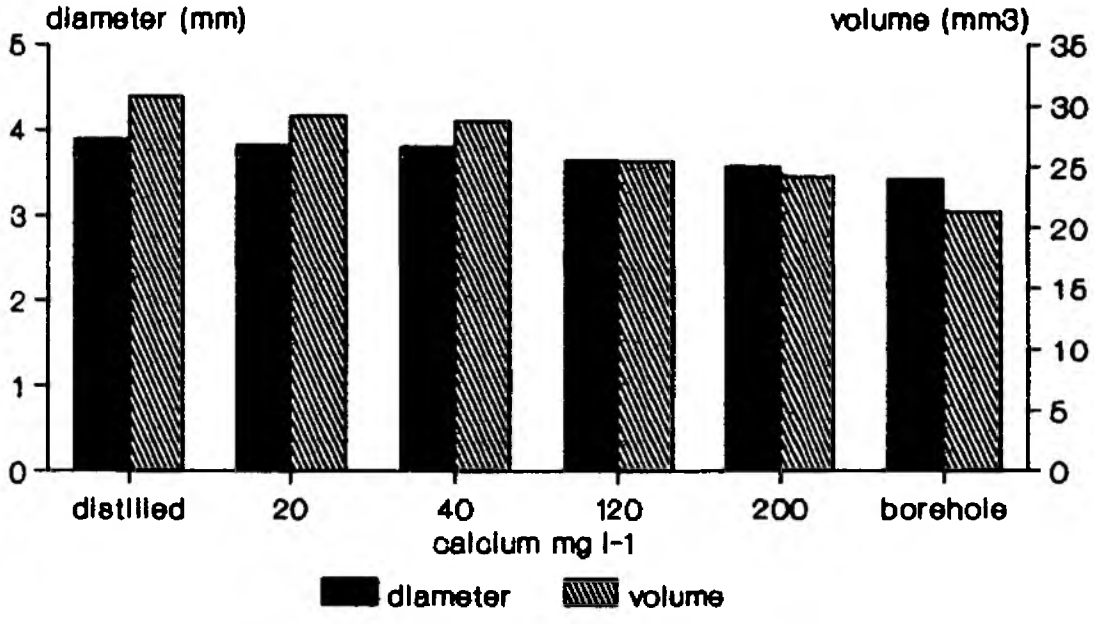
very low, and may have masked any trends that may have occurred due to the effects of different hardening water solutions. It is suggested that this experiment should be repeated very early in the next reproductive season to elucidate whether the use of calcium chloride added to distilled water affords sufficient calcification to the eggs to withstand the trough incubation method. The data on oocyte diameters (Figure 7) from this experiment, however, do suggest that the calcium chloride did affect the ultimate size of the oocyte, probably through osmotic effects on the swelling rate before hardening had been completed.

4 FRY REARING

The alevins produced by induced-spawning methods were reared in trough systems and glass aquarium tanks. Initially, the alevins were fed Artemia for the first three weeks, and from the beginning of week 3, zooplankton were gradually supplemented into the diet. Growth appeared to be excellent, with, albeit a subjective view, the fry in the glass aquarium tanks performing better than those in the trough systems. This may be due to several reasons. Firstly, the fry had a gravel substrate to rest on in the glass tanks, and it was thought that this response may have prevented unnecessary activity and energy loss. Secondly, the trough system may not have been ideal for grayling, due to high flow rates through the trough, lack of gravel to support the developing fry, background trough colour and possible UV light problems. A total of 1120 fry were introduced in two batches into a plankton-rich pond at the NRA fish farm at Snailwell, near Newmarket, at an age of 4 to 6 weeks old. The grayling fry could not be observed almost immediately after their introduction into pond 1. There may be several reasons for their demise. Throughout the early summer months, water temperatures were high, and thus the upper lethal temperature for grayling larvae may have been exceeded during this time. Pond 1 was situated close to the River Snail and the cover afforded by this stream for piscivorous birds, such as kingfisher, may have predisposed pond 1 to high levels of avian predation. The shallow nature of the pond would also have aided predation by such birds. A further possibility was that the grayling may have succumbed to a bacterial infection which was present in grayling larvae maintained at Brampton during the same period. The bacterial problem caused a complete mortality by the end of July in fry at Brampton, but at Snailwell, no dead grayling were observed in pond 1. Feeding by piscivorous birds may, however, have removed any carcasses.

In conclusion, rearing methods were developed to take the alevins from the swim-up stage to being stocked-out in earthen ponds at up to 5 cm in length. Bacterial infections, high temperatures and/or predation accounted for a total

Figure 7 Comparison between grayling egg sizes after hardening in solutions of calcium at different concentrations



mortality of the fry at Brampton and Snailwell. Longer-term rearing on artificial foods was not attempted and this will be discussed with Yorkshire Region NRA who have directed much time on this aspect.

5 DISCUSSION

The research and development work performed in 1989 has been of immense value in understanding the culture requirements of grayling. The *in vitro* trials were designed to determine the likely time to induce ovulations in pre-spawning grayling. The results indicate that grayling ooctyes cannot be stimulated to complete the final maturation processes *in vitro* using exogenous gonadotropin alone. When administered *in vivo*, salmonid and carp pituitary extract effected the completion of final maturation and caused ovulation. Thus, it is possible that a further factor synthesised by the animal itself, which is not present in SPE or CPE, is necessary to complete these processes *in vitro*. The data on steroid production during the *in vitro* experiments, when available, will facilitate this understanding and give an insight into the physiology of an oocyte before ovulation. The presence or absence of certain steroids at critical times will be indicative of the success or failure of the administered pituitary extract to effect the desired changes during final maturation. It will be of great interest to compare the changes in steroid production over the latter stages of vitellogenesis and final maturation, and such data, it is hoped, will indeed indicate the time for successful spawning induction early into the pre-spawning period.

The effects of salmonid pituitary extracts to complete final maturation and effect ovulation in grayling were clearly demonstrated and a reliable protocol for their artificial spawning has been developed. The requirements for the successful incubation of grayling eggs thus produced reached an important level by the end of the transient production period, but further work is required to satisfy the physiological requirements of the developing embryos. Water quality was identified as a potential problem, but after exhaustive tests on the hatchery supply, no parameter has been identified as a major causative agent on egg mortality. It appears that viability and fertilisation may account for some of the observed mortality during the incubation period, mortality not becoming obvious until day 4 to 7. It is impossible to determine the fertilisation rate in eggs being incubated in trough systems until day 4 has been reached, as the sensitiveness of the eggs at this stage precludes examination. However, partitioning of eggs from different females in separate trays eases egg removal if a catastrophic mortality occurs. Attention to detail on stripping times, fertilisation and hardening methodologies, will improve the chances of obtaining fertile eggs for incubation, and

improvements to incubation methodologies are being evaluated at present. The appropriate methodologies for the induction of ovulations in grayling, and subsequent handstripping and egg handling procedures, are given in detail in Appendix 1.

First feeding and rearing grayling fry on Artemia and zooplankton was effective and ensured that the fry crossed the critical period from endogenous to exogenous nutrition without significant mortality problems. Rearing of fry has proved to be more difficult than expected and will be an important area for discussion with Yorkshire Region NRA. Disease problems will be addressed by spawning and rearing fry on two different sites in 1990 and attention to hatchery hygiene will be of paramount importance. The provision of UV steralisation will reduce the likelihood of bacterial infections becoming serious. Pond rearing will be performed in deeper ponds than those used in 1989, and it is hoped that high temperature problems experienced at Snailwell would not recur.

6 FUTURE DEVELOPMENTAL WORK

Several problems for grayling culture still exist and it is hoped that the 1990 season will resolve all of these. To this end, experimental work will be performed on six aspects of this work, as follows.

6.1 Comparison between SPE and CPE with respect to egg viability - the use of CPE will avoid the purchase of SPE which is not used for the spawning of any other species.

6.2 Gonadotropin (CPE/SPE) to promote spermiation in males - spermiation was poor in some males both during and after initial stripping and sperm motility / activity appeared to cease on subsequent stripping occasions.

6.3 Effects of calcium on incubation mortality - calcification of the chorion may be important in the hardening process and unprotected eggs (insufficient calcification) may be more susceptible to incubation mortality in troughs.

6.4 Quantification of ovulation success with the established protocol - the method developed this year must be retested to determine the ovulatory success, and also at different stages during the pre-spawning period.

6.5 Incubation methods - two incubation methods are to be tested, troughs and Zouger jars, at two different fish farms. This will also help to elucidate the effects, if any, of water supply.

6.6 Effect of dietary supplement of carotenoids on egg viability - the tank-maintained broodstock produced eggs lacking in carotenoid pigments and this may have been a major factor in the apparent mortality of the resultant alevins. A project is presently underway to determine the effects of dietary supplementation of Astaxanthin on the pigment levels in the eggs. The pigment levels in oocytes will be monitored through the critical winter period by assay techniques and survival of alevins and fry spawned artificially will be assessed.

SUMMARY

1. A reliable protocol for the induced spawning of grayling using salmonid pituitary extract has been developed, achieving the primary aim of the collaborative project.

2. An important experiment demonstrated the effects of water chemistry on the viability of the developing eggs with particular reference to the hardening period. Calcium ions may be the most important factor.

3. Six areas of work requiring further evaluation have been identified, and these will be addressed in 1990.

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Appendix 1 Induced spawning and fertilisation procedures for the artificial culture of grayling

Induced spawning method

Gravid female grayling should be in pre-spawning condition by mid-March (to be fully assessed in 1990) and therefore receptive to hormonal treatment.

First injection

Time:	0 hours (day 1)
Injectant:	0.6 % saline
Hormone concentration:	2 mg ml^{-1} salmonid pituitary extract (SPE) in saline
First injection:	1 mg kg^{-1} salmonid pituitary extract in saline at 0.5 ml kg^{-1} injectant

Second injection

Time:	48 hours (day 3)
Injectant:	0.6 % saline
Hormone concentration:	6 mg ml^{-1} salmonid pituitary extract (SPE) in saline
Second injection:	3 mg kg^{-1} salmonid pituitary extract in saline at 0.5 ml kg^{-1} injectant

Stripping and fertilisation

Time of first inspection: 120 to 144 hours (days 5 to 6). Eggs should remain viable for up to five days after ovulation.

Fertilisation procedure

Female anaesthetised in benzocaine (1 : 4,000) and dried, with particular attention to the vent and anal fin. Light pressure applied to the abdominal area to effect egg removal, if ovulated. Eggs stripped into a plastic bowl, with care not to allow any water, faeces or blood to pass onto the eggs. Spermiation and sperm motility (under microscope) in male should be checked before fertilisation attempted. Male anaesthetised, dried, and fish stripped of milt in an upsidedown position. Milt accumulating in common pore is removed with a pasteur pipette and placed onto eggs. Desired male:female ratio is 2:1, due to low amount of sperm produced and variable sperm quality (if unchecked). Male discarded after use due to poor viability on subsequent stripping occasions. Fertilisation solution (3 g l^{-1} urea, 4 g l^{-1} sodium chloride) at same temperature, added to eggs and sperm, constituents mixed with feather and rested for five minutes. Eggs are then washed with further additions and

removals of this solution. Water is added to the eggs to effect elevation and hardening of the chorion, eggs then rested in the bowl for at least 60 minutes to allow for this process to be completed. Eggs are then placed in the incubation apparatus (trough, Zouger Jar) for upto 21 days at 10 °C for incubation. Egg picking should be avoided until after gastrulation stage (before day 6). Hatching may commence from day 16 if temperature rises much above 10 °C, but expected time of hatch is from day 21 onwards. Newly hatched alevins lie on the trough bottom for up to 10 days and then swim-up after yolk sac absorption. First-feeding with Artemia and zooplankton should commence immediately after swim-up. Feeding response to live food is good.

NEIL MAC