2. Chapter Two: General Materials and Methods

2.1. Experimental Embryos

All laboratory trials utilised diploid brown trout (*Salmo trutta*) embryos sourced from Allenbrook Trout Farm, Dorset, UK. Atlantic salmon (*Salmo salar*) embryos used as the experimental fish in field trials were supplied by the Environment Agency Cynrig Hatchery, Brecon, Wales. Brown trout embryos used as the experimental species in field trials were from Allenbrook Trout Farm.

Eye-pigmented embryos, 14 days post-fertilisation (dPF), used in experiments described in Chapter 3 and Chapter 4, and green eggs (pre-fertilised), as used in Chapter 3 and Chapter 5, were transported in pre-cleaned plastic containers within ice cooled boxes (Figure 2.1). Polystyrene supports separated the ice from direct contact with the eggs to prevent any significant temperature change. Milt was transported in a similar manner but was contained within polystyrene cups.

Figure 2.1. *Salmo salar* eggs from Cynrig hatchery before transportation.

2.2. Fertilisation Protocol

Experiments involving contaminant exposure for the full incubation cycle required fertilisation immediately prior to exposure (0d). Green eggs collected were always sourced from more than one adult female and mixed before fertilisation. Milt stripped from at least

two adult males was combined and gently stirred using a Pasteur pipette. The fish farmer would check the milt after stripping to ensure suitable abundance. Eggs and milt sourced from Cynrig hatchery used wild Atlantic salmon from the River Cynrig, a tributary of the River Usk, Brecon. At least three females and males were caught and stripped for the eggs and milt so that the gametes were of mixed parentage. The use of gametes from more than one male and female was to account for the possibility that an adult fish could be sterile.

Eggs were carefully counted into a clean glass beaker using a plastic tea strainer and Pasteur pipette to minimise handling and prevent mechanical damage. Eggs were fertilised in appropriate batches with equal amounts of milt (1ml). Once the milt was added (Figure 2.2), the eggs were gently stirred for 30 seconds with a Pasteur pipette. Fresh water (100ml) was added to the mixture to activate the milt (it is imperative that water does not come in contact with the milt before it is needed for the fertilisation process). After 4 minutes, the milt was washed away with one exchange of clean water. The fertilised embryos were now ready to be used in experiments.

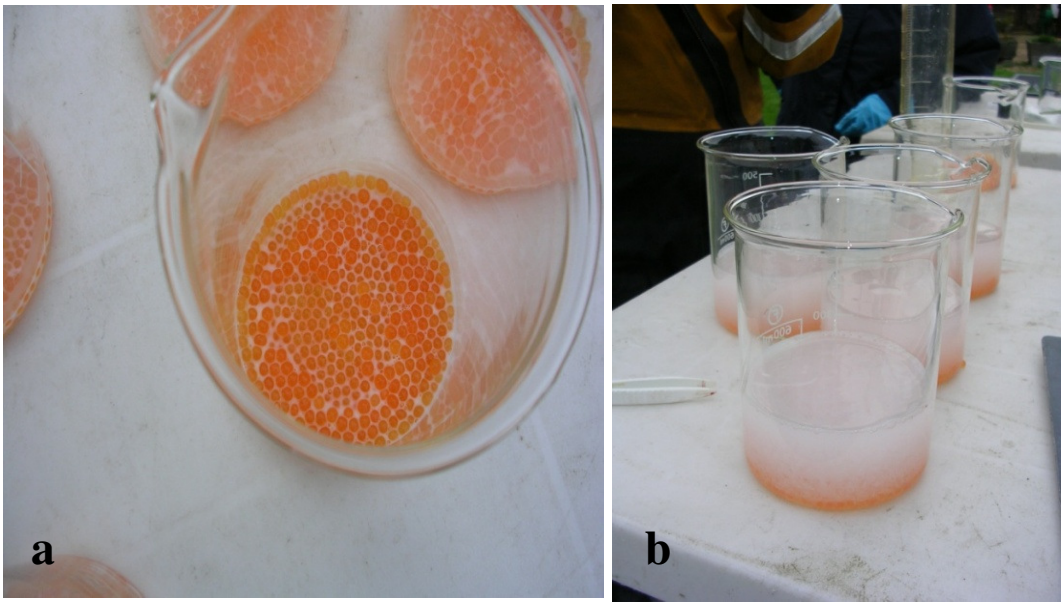

Figure 2.2. Addition of milt (a) and water (b) to fertilise the green salmonid eggs.

2.3. Experimental Conditions

All regulated procedures were carried out under Personal Licence (PIL80/9495) from the UK Government Home Office under The Animals (Scientific Procedures) Act 1986. Although the very early life stages of salmonids are not covered by the Act, once the hatched alevins form a gut and can become capable of independent feeding, any procedure then become regulated. Therefore any experiment including this stage of development was performed in a designated area and covered by both a Project Licence (PPL80/1753) and carried out by those with appropriate Personal Licences (PIL). The health and welfare of the organisms involved in any of this research was therefore ensured. All alevins and fry were killed using a Schedule 1 procedure, a fatal dose of 2-phenoxyethanol (4ml/l) followed by destruction of the brain.

For all pesticide exposures in water the embryos were kept in complete darkness as much as possible as to represent the conditions in a natural redd. For sediment-bound pesticide experiments it was only necessary to have a controlled photoperiod for the emergence stage. The photoperiod reflected the actual daylight hours, and represented the natural conditions for the fry. The photoperiod for each experiment was controlled by an electromechanical time switch (Sangamo S250/Q550).

2.4. Artificial Freshwater (AFW)

AFW used for laboratory trials (Chapter 4) was prepared according to guidelines for US EPA soft water and comprised: 1.2g $MgSO_4$, 1.92g $NaHCO_3$, 0.08g KCL dissolved in 1l reverse osmosis (RO) water and made up to 19l. This solution was then aerated for 30 minutes before addition of 1.2g $CaSO_4.2H_2O$ dissolved in 1l RO water (US EPA 1994). RO water was supplied by an integral pure water system (Ondeo, Purite Select, UK).

2.5. Artificial Sediment

The procedure for producing artificial sediment for laboratory trials (Chapter 5) was adapted from Greig *et al*., (2005). Newplast modelling clay (Newclay Products Ltd,

Newton Abbot, UK), was combusted at 450ºC for five hours prior to use to remove any organics. This was to remove any organic compounds and associated oxygen demands. It was then necessary to mechanical macerate the clay using a pestle and mortar to achieve the desired sediment particle size i.e., fines. Toxicity information supplied by Newclay Products Ltd and measured under BS EN 71-3, indicated undetectable levels of common metaliferous toxins (e.g., mercury, lead, cadmium, copper, arsenic) in the modelling clay. The raw material for the modelling clay is sourced from Devon and mined by WBB minerals.

2.6. Standard Solutions (for dosing and calibration)

Stock solutions of contaminants were made up using pure compounds (QMX, UK) weighed on a four figure balance (Fisherbrand PS-100) on aluminium foil and dissolved in either acetone (HPLC Grade, Fisher, UK) or methanol (HPLC Grade, Fisher, UK). Solutions were placed in an ultrasonic bath at room temperature for 10 minutes to ensure that the compounds were dissolved. Stock solutions were kept in amber volumetric flasks and refrigerated at 4°C.

2.7. Nutrient Analysis of Field Sites

Water Samples collected from the field sites were analysed for Total Ammonia Nitrogen (TAN), nitrate, nitrite, phosphate and sulphate using the commercial Palintest® spectrophotometer. All water samples were firstly filtered through a GFC filter (Fisher, UK) and then analysed using the Palintest reagent kits (Appendix 1).

2.8. Analysis of Embryos and Early Life Stages

2.8.1. Morphometic Measurements

Physiological effects on embryos from the trials were monitored by egg weight and diameters, and the survival rates were also recorded. Eggs were carefully blotted dry

before being weighed on an analytical balance with an accuracy of ±0.0005g (Denver Instrument, SI-403). Diameters (Figure 2.3) were measured using digital calipers (Mitutoyo) accurate to 2 decimal places.

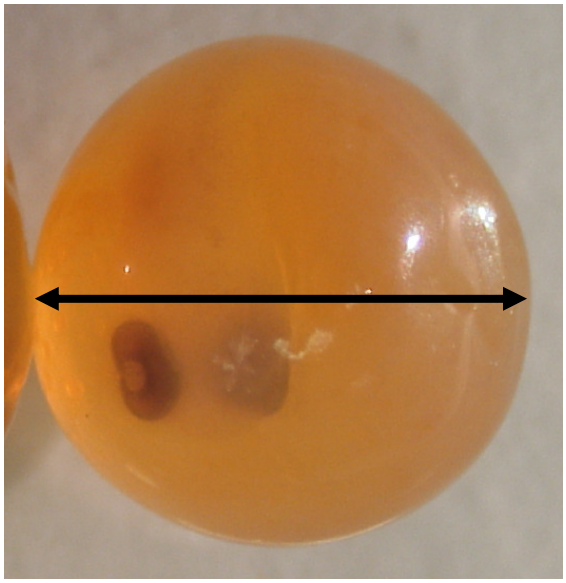

Figure 2.3. Salmonid egg indicating the measurement of diameter.

From the weight measurements it was also possible to calculate volume (V) and surface area (SA) and thus SA:V ratios;

a) V and SA calculated by mass;
   i) Egg volume (cm^3) as $\boldsymbol{V = mass\ (g) - 0.00884}$ (Fleming & Ng, 1987);
   ii) Surface area (cm^2) as $\boldsymbol{SA = 4\pi(3V/4\pi)^{2/3}}$ (Einum et al., 2002)

Each alevin was weighed upon hatch, as well as the total length measured using digital calipers (Figure 2.4.).

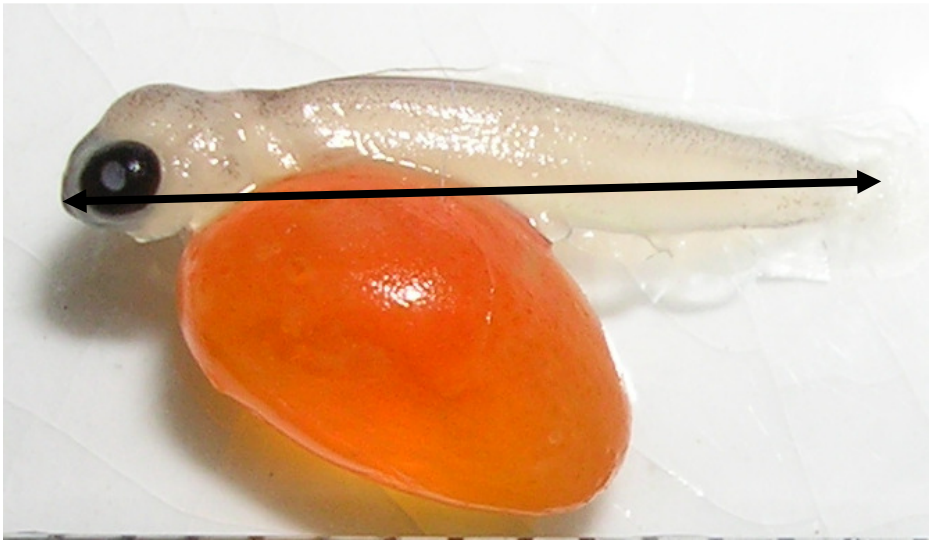

Figure 2.4. Alevin length measurements were taken as total length.

The yolk sac was removed carefully (see Figure 2.5) using the point of a sharp scalpel and weighed independently of the alevin body. The % of the total alevin made up of body and the % of the total alevin made up of yolk sac were then calculated.

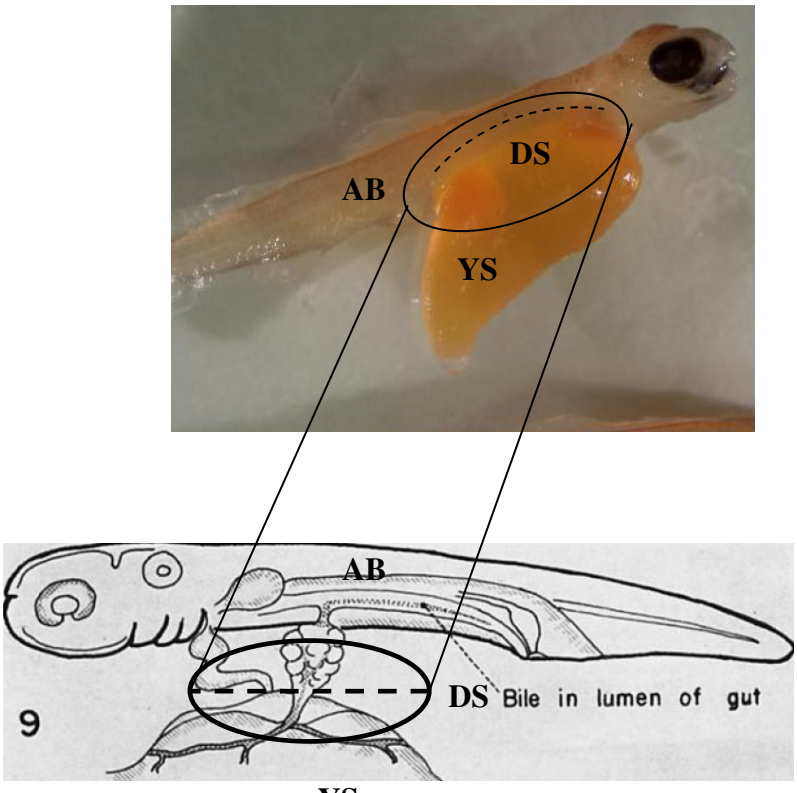

Figure 2.5: Alevin of *Salmo salar*, showing site of dissection of yolk sac for analysis of % yolk of the total body weight. YS- Yolk Sac; AB- Alevin Body; DS- Dissection Site. Adapted from Pelluet (1944).

2.8.2. Observations of Malformations

During sampling, any signs of malformations were noted. These included lordosis (curvature of the spine), haemorrhaging and yolk sac oedemas (Figure 2.6).

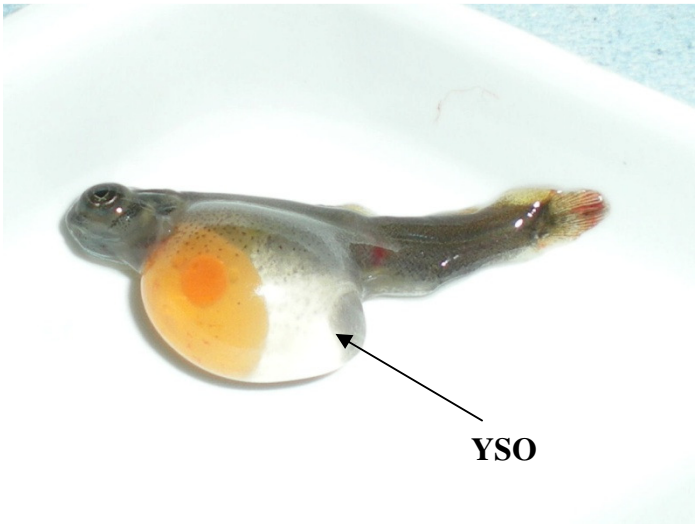

Figure 2.6. An example of a malformation, a yolk sac oedema (YSO).

2.8.3. Fixatives

Once eggs and alevins had been measured and weighed, they were transferred to the appropriate fixative. For standard fixation, Neutral Buffered Formalin (NBF), was used (Appendix 2), for the fertilisation scoring (Chap. 4) Stockard's Solution (Appendix 2) was used.

2.8.4. Dry Weight and Water Content Analysis

For the dry weight analysis, frozen eggs were thawed and blotted dry then placed in a pre-weighed 1.5 ml micro centrifuge tube. The tube was then reweighed with the egg and the total weights recorded. These tubes were then heated in an oven at 60^0C for 72 hours (to constant weight) and then allowed to cool in a desiccator. From reweighing the micro-centrifuge tubes the egg dry weights and percentage water contents were calculated and recorded.

2.8.5. Ninhydrin-Positive Substances (NPS)

NPS measurements give an indication of metabolism in the embryos. Concentration of NPS is used as a proxy of Free Amino Acids (FAA) and levels are found to increase during normal development of Atlantic Salmon, as protein is broken down into FAA (Srivastava *et al*., 1995). The method was adapted from Srivastava *et al.* (1995) and dry weight of the eggs was measured prior to NPS analysis. Three pre-weighed blank microcentrifuge tubes were also heated with the samples to eliminate the possibility of weight change due to the heating of the tubes. 1ml of 5% Tricholoroacetic (TCA) was added to each dried egg in the microcentrifuge tube and left at room temperature (RT) for 7 days or until the egg had digested. It was necessary to mechanically break up the embryo tissue using a glass homogeniser halfway through the digestion period in order to ensure penetration of the chorion. The microcentrifuge tubes were centrifuged (Eppendorf, Centifuge 5810R) at 6500rpm for 10 minutes. The supernatant was kept for the NPS measurements.

A stock solution (2mM) and standard solutions were prepared as described in Appendix 3. TCA extract and duplicate standard solutions, 20µl and 100µl respectively, were transferred to a thick glass test-tube and 80µl of distilled water was added only to the TCA extract. Ninhydrin Reagent (Sigma, UK) was added to each test-tube (300µl) and vortex mixed. All tubes were heated at 100ºC for 20 minutes, after which 3ml 50% Ethanol was added to each tube. All the test tubes were consequently vortex mixed, transferred to a straight sided 4ml cuvette (Fisher, UK) and absorbance read at 570nm on a spectrophotometer (Novaspec II).

2.8.6. Protein

Protein measurements can also be used as another indication of metabolism. The remaining pellet once the TCA supernatant was removed (TCA extract) was resuspended in 1ml of distilled water and centrifuged at 6500rpm for 10 minutes. The supernatant was discarded and the pellet resuspended in 1ml of 1N Sodium Hydroxide (NaOH) and left at RT for 7 days for the proteins to digest. The samples were then centrifuged at 6500rpm for 10

minutes to pellet the undigested chorion and the supernatant was kept for protein measurements.

Stock and standard solutions were prepared as described in Appendix 4. 50µl of the NaOH extracts and 50µl standard solutions were transferred into glass test tubes. 3ml of Biuret Reagent (Appendix 4) was added to all test tubes, vortex mixed, and incubated at 37ºC for 30 minutes. All standards and samples were transferred to straight sided 4ml cuvettes and absorbance read at 540nm on a spectrophotometer (Novaspec II).

2.8.7. Chloride Analysis

The protocol for chloride analysis was adapted from Barrett *et al*. (2001). Each egg was placed in a 1.5 ml microcentrifuge tube and 500 µl of 1N Sulphuric Acid (H_2SO_4) was added and left at RT for 7 days. After 3 days the embryos were mechanically broken up with a glass homogeniser and left for the remaining days. Embryos were then centrifuged at 10000rpm for 10 minutes. Supernatant was transferred into a new microcentrifuge tube ready for analysis.

Samples were analysed for choride using a chloride meter (Jenway PCLM3). The chloride meter was first buffered with a combined acid buffer (Sherwood, UK) and then calibrated using the 100mmol/l Standard (Jenway, UK) and then 20µl of each sample was used for analysis and results recorded in units of nEq Cl^-/mg egg.