The Effects of Oxygen Depletion due to Upwelling Groundwater on the Post-Hatch Fitness of Atlantic Salmon (*Salmo salar*).

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Abstract

The conditions experienced by incubating Atlantic salmon (*Salmo salar*) eggs are strongly influenced by hyporheic exchange. In some rivers, periods of intense groundwater upwelling can reduce oxygen levels in the incubation zone to 0% saturation. The present study investigated the effect of oxygen sags on the post-hatch fitness of Atlantic salmon. A laboratory experiment allowed fine-scale control of oxygen concentrations to replicate those induced by low oxygen groundwater in rivers. Extreme oxygen sags in the earlier stages of embryo development resulted in a developmental lag with alevin hatching later and at an underdeveloped state. At the latest stages of development, oxygen sags caused premature hatching of severely underdeveloped alevin. These findings combined with a review of the literature suggest post-hatch survival of embryos exposed to groundwater induced hypoxia will be lower due to predation and poor competitiveness.

Keywords: Groundwater, hyporheic exchange, hypoxia, post-hatch, sublethal, biometrics.

Introduction

Despite their ecological and economic importance (e.g. Carss *et al*. 1990; Everard 2004), Atlantic salmon (*Salmo salar*) are in decline. Many populations throughout Northern Europe have been extirpated (Parrish *et* al. 1998) and in 2014, the number of mature salmon returning to the United Kingdom to spawn was just 41% of the 1970s average (ICES 2015). The proposed reasons for this decline include overfishing (Slaney *et al*. 1996), reduced marine survival as a result of changes to climate patterns (Friedland *et al*. 2000; Otterson *et al*. 2001) and reduced incubation success due to unfavourable conditions (Chapman 1988). Incubation success can be severely limited by an insufficient oxygen supply during (Greig *et al*. 2007).

Atlantic salmon deposit eggs in nests in the riverbed known as redds which, in ideal conditions, provide the eggs with a continuous supply of well-oxygenated water (Greig *et al*. 2007). However, a range of natural and anthropogenic factors can reduce oxygen delivery (Sear *et al*. 2008). For example, sediments can infiltrate salmonid redds, reducing interstitial velocity and therefore delivery of oxygen to the eggs (Greig *et al*. 2005; Sear *et al*. 2008), resulting in high mortality rates (Kemp *et al*. 2011).

Recently, the importance of hyporheic exchange and the effect of groundwater (GW) - surface water (SW) interactions on oxygen delivery to salmonid eggs has been demonstrated (Malcolm *et al*. 2003; Sear *et al*. 2014). GW can be severely oxygen depleted (Malard & Hervant 1999), so its presence in the incubation zone will reduce oxygen availability to salmonid eggs. Field data suggests that deoxygenated GW upwelling is widespread. Indeed, of 12 salmonid spawning sites studied in northern Europe, 11 showed evidence of oxygen depletion as a result of GW upwelling (Greig 2004; Malcolm *et al*. 2006; Soulsby *et al*. 2009; Burke 2011; Bateman 2012; Schindler Wildhaber *et al*. 2014; Sear *et al*. 2014).

Low oxygen levels during incubation can induce sublethal reductions of embryo fitness such as altered hatch timing (Youngson *et* al. 2004; Roussel 2007), increased frequency of deformities (Alderdice *et al*. 1958) and reduced alevin length and mass at hatch (Youngson *et al*. 2004; Geist *et al*. 2006). Only Youngson *et al*. (2004) considered the impact of deoxygenated GW on post-hatch fitness of Atlantic salmon, but their findings were based on intermittent (twice monthly) readings of oxygen levels in the field. Higher resolution studies (e.g. Malcolm *et al*. 2006) have shown that oxygen levels in the incubation zone can fluctuate over very short temporal scales. This demonstrates the need to continuously monitor oxygen levels throughout the incubation period and could help to identify developmental stages where the embryo is most sensitive to GW induced hypoxia.

Human interventions such as river water abstraction (Hancock 2002) and log-step construction (Schindler Wildhaber *et al*. 2014) can increase GW upwelling, so understanding the effects of oxygen sags on incubating Atlantic salmon will aid river management. In addition, deoxygenated GW appears to be present in many locations hosting Atlantic salmon populations (Greig 2004; Malcolm *et al*. 2006; Soulsby *et al*. 2009; Burke 2011; Bateman 2012; Schindler Wildhaber *et al*. 2014; Sear *et al*. 2014), so these effects could be widespread. Therefore, it is useful to understand the impacts that such oxygen depletion events might have on embryonic Atlantic salmon.

This study investigated the effect of three similar sequences of oxygen depletion events, comparable to those caused by GW upwelling, on the post hatch fitness of Atlantic salmon embryos. In particular, the effects of these sags on (1) hatch timing and (2) post-hatch biometrics was observed. In addition, the importance of the timing of these sequences of oxygen depletion events on the magnitude of each response was monitored.

Materials and Methods

Egg sources

To account for variation between locations, eggs were collected from four sites within the United Kingdom (Fig. 1). Except for those of the farmed fish, all eggs and milt were taken from adults naturally returning to their natal stream:

1. River Burn hatchery, River Ure, North Yorkshire.
2. Kielder hatchery, River South Tyne, Cumbria.
3. Kielder Hatchery, River Rede, Northumberland.
4. Commercial farm, Argyll and Bute, Scotland.

As the focus of the present study was English salmon rivers, the first three sites were selected as they were locations with a sufficient broodstock of wild fish to permit egg samples. In addition, the farmed eggs were selected as they represented eggs taken from an optimum incubation environment. Due to limitations on the number of eggs that could be donated, eggs were obtained from a single maternal fish from each location. During data analysis, the eggs of all the fish were combined. This total of four maternal fish was greater than similar studies (Malcolm *et al*. 2003; Youngson *et al*. 2004; Roussel 2007) and was sufficient to provide statistically robust data.

In all cases, gametes were extracted from adult fish by hand on the morning of 1st December 2014 into clean and dry plastic containers. Eggs were submerged in coelomic fluid to prolong their viability (Bonnet *et al*. 2003). Containers were immediately oxygenated and placed into chilled containers before being transported to the Chilworth Spawning Habitat research facility at the University of Southampton. Transportation of unfertilized gametes results in greater survival than fertilized gametes (Jensen & Alderdice 1983). Further, fertilization rates

of 95-100% have been observed for up to five days post-extraction when gametes are stored in this way (Jensen & Alderdice 1984).

Research facility and oxygen control

Research was conducted in a continuous recirculating, tap water fed system (Fig. 2) described in detail in Sear *et al*. (2016).

Eggs were fertilised in the facility on the morning of 2nd December 2014 following procedures described by Whitney *et al*. (2013). Eggs of each maternal fish were divided into 5 groups of 300. Each of these groups was exposed to one of five oxygen treatments described below. To account for variation between egg chambers, each group was further sub-divided into 3 replicates of 100 eggs each. In addition, a batch of 200 eggs per maternal fish was held in reserve.

Eggs were left to acclimate to the conditions until daily mortality rates fell below 1%. This occurred after three days, or 22 Degree Days (DDs). During the acclimation period, all eggs within the containers that died were removed and replaced with a live egg from the reserve batch of the corresponding maternal fish source. After 22DDs, those that died were removed but not replaced as they had not faced the same level of oxygen stress as the test eggs.

Test eggs were placed into egg chambers made of perspex (25cm x 15cm x 12cm, at a water depth 7cm) lined with artificial grass, a suitable substrate for incubation (Hansen & Møller 1985). All containers had a lid that could be easily removed to minimise disturbance during sampling. Boxes and lids were opaque black to ensure that light damage (Flamarique and Harrower 1999), was minimised. Fine mesh netting was attached around the outflow pipe of each egg box to eliminate the risk of post-hatch alevin being lost.

The bulk flow rate through each egg chamber was maintained at 150cm h^{-1} ± 3.6% to ensure it was not a limiting factor in the oxygen supply to incubating embryos (Greig *et al*. 2007).

Oxygen levels were controlled in cylindrical oxygen modification chambers (height 140cm, diameter 50cm; Fig. 2) through the addition of nitrogen. Compressed nitrogen gas was transported through flexible tubing of inner diameter 6mm (RS®) to acrylic flowmeters (Omega®). The flowmeters allowed precise control over nitrogen flow, and thereby oxygen levels. Nitrogen gas was transported from the flowmeters to a single fine-bubble air diffuser (Track Lock®), one of which was placed into each oxygen modification chamber, including the control. Oxygen modification chambers were totally sealed, with the exception of perforations for the water inlets, nitrogen inflow and oxygen probes. This provided greater control over oxygen levels by minimising atmospheric oxygen exchange.

Aandera® optodes recorded temperatures at 1 minute intervals throughout the experiment and the mean over ten minutes was logged on a Delta-T data logger. Average recorded temperature was 7.62ºC (min: 4.94ºC; max: 10.32ºC). The continuous monitoring of temperature allowed precise calculation of the embryonic developmental stage in DDs (Gorodilov 1996).

Daily water quality checks were taken to measure ammonia, nitrate, nitrite, copper, phosphate and pH levels. Readings were consistently below critical levels recorded for incubating salmonids (Sear *et al*. 2016).

Oxygen Treatments

Five different treatments were established to determine the relative impacts of various oxygen regimes on post-hatch fitness of Atlantic salmon embryos (Table 1; Fig. 3 A - E). This involved two continuous treatments (Table 1; Fig. 3 A and B) where target oxygen levels were consistent throughout the experiment;

A. Control. Oxygen saturation was maintained close to maximum. Mortality rates in this treatment were used to calculate relative sensitivities to hypoxia of the other treatments;

B. Chronic. After the period of acclimation (22DDs), oxygen levels were maintained at approximately 60%. This enabled comparisons of the response of Atlantic salmon embryos to prolonged mild hypoxia compared to treatments mimicking more extreme, sporadic hypoxia induced by deoxygenated GW.

Three separate oxygen sag treatments (Table 1; Fig. 3 C - E) were also developed based on analysis of available datasets recording the effect of GW upwelling on the oxygen content of salmon redds (Greig 2004; Malcolm *et al*. 2006; Soulsby *et al*. 2009; Burke 2011; Bateman 2012; Schindler Wildhaber *et al*. 2014; Sear *et al*. 2014). The sag treatments consisted of a sequence of 10 oxygen depletion events (pulse) lasting 24hrs. Each pulse was separated by a period of oxygen recharge of the same duration. The mean number of pulses, pulse intensity and pulse duration for the Atlantic salmon incubation period were calculated from the raw datasets. These were used to configure the oxygen regimes for each sag treatment. Each sag treatment was consistent in the number of pulses, pulse intensity and pulse duration but differed only by the time that the sequence of pulses began (Table 1; Fig. 3 C-E). This was altered to mimic annual variation in rainfall and therefore hyporheic exchange patterns. Throughout each sag treatment, pulse intensity gradually increased to simulate the increasing dominance of GW in the incubation zone (Table 1; Fig. 3 C - E). Sag treatment start times are displayed in DDs to enable easier identification of embryonic development periods most sensitive to low oxygen levels. Treatments C-E were the sag treatments and were categorised as follows:

C. Early Sags. Pulse sequence described above commencing at 134DDs;

D. Median Sags. Pulse sequence described above commencing at 229DDs;

E. Late Sags. Pulse sequence described above commencing at 317DDs.

Oxygen levels were continuously monitored at 1 minute intervals and logged to a Delta-T logger every 10 minutes throughout the investigation using Aandera® 4175 optodes.

Re-oxygenation occurred between oxygen modification chambers and the egg chambers. This was noted in preliminary investigations and was accounted for by reducing oxygen to levels below the target values. The rate of re-oxygenation was proportional to the amount of oxygen initially removed in the oxygen modification chamber and could be estimated using the following equation:

$$E_o = M_o + \left(\left(\frac{C_o - M_o}{100}\right) \times 10\right)$$

Where:

E_o = the estimated oxygen level in the egg chambers;

M_o = the oxygen concentration in the oxygen modification chamber;

C_o = the oxygen level in the control.

Daily spot checks within egg chambers were conducted using a handheld oxygen probe (YSI® proODO) to ensure oxygen concentrations were close to the target values described in table 1 and the estimate calculated using equation 1. Oxygen levels in the egg chambers were within 2.7% (min: 0%; max: 12.34%) of estimated values on average with greater fluctuation observed between egg chambers for the lower oxygen treatments (Fig. 3 C - E).

Measurement of sublethal effects

Daily checks of the presence of hatched alevin were conducted. To increase data resolution, the frequency of these checks was increased to twice daily during the period of peak hatching (434DDs onwards). The number of hatched alevin at each sampling time was recorded. All hatched alevin were immediately taken from the system and euthanised using 2-phenoxyethanol solution and preserved in 4% formalin solution (Burke 2011).

To sample post hatch biometrics, alevin were removed from the formalin and thoroughly rinsed in deionised water. Initial observations were conducted to detect the presence of developmental abnormalities. These were present in less than 0.4% of sampled individuals and their frequency did not vary among treatments, so they were not included in data analysis.

The fork length of a sample of wet alevin from each treatment was measured using a Nikon E100 microscope at 50x magnification. To account for errors, alevin were re-measured and differences were <0.1mm.

Alevin were then oven-dried at 60°C for 48hrs (Rombough 1994) and measured for total mass (yolk sac plus body). The yolk sac was subsequently detached and the body was weighed to determine its contribution to total mass. All mass measurements were conducted on a Mettler Toledo AB204-5 balance accurate to 0.1µg.

From the completion of dehydration to measurements of alevin mass, there was the potential for rehydration of the alevin from atmospheric moisture. To determine whether this resulted in a significant increase in mass, a small sample of alevin of each treatment/population group was measured immediately after dehydration and subsequently at 2-hour intervals over a total period of eight hours. Over this time, the total combined mass increased by 0.77%. To ensure that this effect was minimised, all samples were measured within two hours of removal from the dehydrator.

The developmental state of alevin in newly hatched alevin was measured by counting the number of caudal fin rays (CFRs) present (Gorodilov 1996). The first CFR is present when anal and dorsal fin formation begins (approx. 300DDs) and continues until a total of 20-21 CFRs are present in post-hatch alevin (Gorodilov 1996). CFRs are formed at equal intervals (Gorodilov 1996), so they provide a useful indicator of developmental stage.

Number of CFRs present was counted under a Nikon E100 light microscope at 100x magnification (Fig. 4).

As alevin were removed from the system and euthanised within 12hrs of hatch, variable hatch timing meant that raw biometric data could not be used to directly compare the developmental state of alevin across treatments at a specified time. Therefore, work by Gorodilov (1996), who used the number of somite pairs present in the embryo as a key indicator of developmental stage, was used to estimate alevin developmental state at a time when all individuals had hatched. Somite pairs are formed during the division of axial strips of the mesoderm (Gorodilov 1996). After the first CFR is formed, the rate of somite pair formation is directly proportional to the rate of CFR formation (1 CFR = 8.33 somite pairs). The rate of somite pair formation could be estimated using the following formula:

$$\lg \tau_t = C + at + bt^2$$

Where:

τ_t = Length of time required to form one somite pair at given temperature t

C = 3.0984

a = -0.0967

t = temperature in ºC

b = 0.00207

This information was used to estimate the number of CFRs of each individual at a time when all individuals from all treatments had hatched. (526DDs), thereby allowing direct comparisons between alevin of different treatments at a single time.

Statistical analysis

There were minimal differences between the eggs of the different maternal fish in terms of their sublethal responses to the different treatments. Therefore, before statistical analysis, the

raw hatching and biometrics data for the eggs of the four maternal fish was combined into a single group for each treatment. This enhanced comparative analysis between treatments.

Hatch timing

Kaplan-Meier (Kaplan & Meier 1958) estimators were used to determine the hatch rates of each treatment. This provides a value for the frequency of hatching for each treatment per cumulative 1000DDs (i.e. the cumulative total of the number of DDs that all the eggs of each treatment combined experience. For example, 100 eggs experiencing 10DDs would experience a cumulative total of 1000DDs). This allows pair-wise comparisons among treatments by producing a ratio that compares the incidence of hatching of two separate treatments.

A value for the hatch rate for each treatment was produced using the following formula:

$$\mu = \frac{h}{E}$$

Where:

μ = Hatching rate

h = Number of eggs that hatched

E = Total exposure time (DDs).

Relative risks of hatching (RRs) of two treatments within each egg group were obtained by taking the hatching rate from the formula above of the two target treatments and dividing them by one another:

$$RR = \frac{\mu_{t1}}{\mu_{t2}}$$

Where:

RR = relative risks

μ_{t1} = hatch rate of treatment 1

μ_{t2} = hatch rate of treatment 2

Two-tailed log-rank tests were performed to determine the statistical significance of all pair-wise comparisons.

During the experiment, a total of 414 (6.9%) eggs died before hatching (Bloomer *et al*. in submission). Mortality was regarded as a random censoring event, independent from hatching. Here hatching incidence and the total time of observation figures are reported from 350DDs. The threshold of 350DDs is based on existing literature that demonstrates no evidence of Atlantic salmon hatching before this stage (Gorodilov 1996), and is supported by the Kaplan Meier hatching curves presented here. Analysis from this point onwards used the enhanced data resolution, enabling more detailed comparisons among treatments. All hatch rate analysis was conducted on the statistical software Stata IC 12.

Alevin biometrics

A mean value for alevin fork length, mass, number of CFRs at hatch and number of CFRs at hatch completion for each treatment was calculated

To compare differences among treatments for all biometrics, one-way ANOVA tests were used. When statistically significant ($p<0.05$) differences among groups were observed, post-hoc Tukey's tests were conducted to determine between which treatments these differences were observed.

All alevin biometric analysis was conducted on the statistical package SPSS 21.

Ethics Statement

The use of animals in this experiment was reviewed and accepted by the University of Southampton Ethics and Research Governance Online (ERGO) panel. All organisms were cared for in accordance with the Guide to the Care and Use of Experimental animals (www.ccac.ca).

Results

Hatch Timing

The first egg hatched at 380DDs and the last at 526DDs. Eggs exposed to the early and median sags treatment showed delayed hatch relative to the control (Fig. 5), reflected by lower incidence of hatching per 1000DD (Table 2, 3). Similarly, alevin of the early sag treatment hatched earlier than those of the chronic treatment.

Eggs exposed to the late sags hatched earlier than all other treatments (Table 2, 3).

Biometrics

Mean alevin fork length differed among treatments (one way ANOVA - $F(4, 908) = 64.191$, $p<0.001$). Alevin of the late sags were approximately 1mm smaller than alevin of the other treatments (Table 4) ($p<0.001$ for all comparisons).

Overall, the total dry body mass of post-hatch alevin was 48.17 ± 8.71mg and there were no differences among treatments.

Mean percentage body mass differed among treatments ($F(4, 908) = 139.628$, $p<0.001$). Alevin of the late sags treatment had a lower percentage body mass than those of the other treatments (Table 4) ($p<0.001$ for all comparisons). Percentage body mass of the control alevin was greater than the chronic ($p<0.001$) and median sags ($p = 0.019$) treatments.

Mean developmental state differed among treatments ($F(4, 662) = 312.300$, $p<0.001$). Alevin of the control were more advanced than those of all other treatments (Table 4) ($p<0.001$ for all comparisons) at hatch. By contrast, alevin hatching in the late sags treatment had fewer CFRs at hatch than alevin of all other treatments ($p<0.001$ for all comparisons).

At the time when all individuals had hatched (526DDs), the estimated number of CFRs present in alevin of all three sag treatments was very similar (Table 4). Significantly more CFRs were present for alevin of the control than all other treatments ($p<0.001$ for all comparisons).

Discussion

The presence of Groundwater in Atlantic salmon redds can result in severe oxygen sags (e.g. Malcolm *et al*. 2006; Soulsby *et al*. 2009; Sear *et al*. 2014). This study showed that such sags can alter alevin hatch timing and biometrics and that these impacts vary depending on the timing of the oxygen stress.

Hatch timing

Oxygen sags during incubation resulted in a substantial shift in the hatch timing of Atlantic salmon embryos. The direction of this shift varied and was determined by the timing of the oxygen sags. Eggs exposed to the early and median sags hatched late and those exposed to the late sags hatched prematurely. Studies looking at the effects of longer-term chronic oxygen depletion have observed similar patterns (Alderdice *et al*. 1958; Oppen-Berntsen *et al*. 1990; Youngson *et al*. 2004). These responses are considered to be adaptive to maximise immediate survival in suboptimal conditions.

Delayed hatch of embryos exposed to hypoxia in earlier stages of development is associated with the ability of the embryo to modify oxygen uptake relative to availability (Hamor and Garside 1976). The oxygen consumption and heart rate of incubating teleosts reduce in hypoxic conditions (Hamor & Garside 1976; Czerkies *et al*. 2002). This leads to a reduction in metabolic rate and a developmental lag that is responsible for the delayed hatch in the present study.

The late sags treatment triggered a different embryonic response due to physiological changes during incubation. In addition, the number of hatching gland cells (HGCs) reaches a maximum and the amount of hatching enzyme (chorionase) contained within the HGCs also increases (Luczynski and Ostaszewska 1991). The oxygen stress induced by the late sag treatment, coupled with the maturation of the embryonic hatching apparatus at this time

facilitates premature hatching. This mobilises the embryo, enabling escape from unfavourable conditions (Czerkies *et al*. 2001).

Embryos exposed to the chronic treatment showed similar hatch rates to the control. The oxygen content of this treatment was consistently close to the upper limits of the critical levels described for Atlantic salmon (Louhi *et al*. 2008), so it is likely that this was sufficient to ensure the metabolic rate was not restricted.

Biometrics

Alevin of the late sag treatment differed most from the control. They had the shortest body length, the lowest level of yolk-sac absorption and the fewest caudal fin rays. By contrast, alevin of the early and median sags were of similar length to the control, although they did differ in terms of the other measured biometrics.

The finding that hypoxia in earlier stages of development had little effect on alevin length at hatch contrasts with previous work (e.g. Silver *et al*. 1963; Hamor & Garside 1977; Miller *et al.* 2008). This is probably due to the fact that extreme hypoxia in these other studies persisted for up to 7 days, much longer than the present experiment. In addition, the extended incubation time of the individuals exposed to the early and median sags presumably enabled compensatory growth. Alevin exposed to the late sags were smaller than their conspecifics of all treatments at the time of hatch. It is likely that this is a result of a combination of developmental lag caused by the earlier pulses of the sag treatment and premature hatch as a result of the stress response to extreme hypoxia described above.

In contrast with other studies, there were no differences among treatments in terms of total mass at hatch. Shumway *et al*. (1964) found that chronic hypoxia gave rise to alevin of significantly reduced total mass. Further, fieldwork conducted by Youngson *et al*. (2004) showed that eggs buried deeper in the hyporheos and more strongly exposed to hypoxic groundwater weighed less. However, Geist *et al*. (2006) found no differences in total mass

between alevin of eggs raised in hypoxic and normoxic conditions. In the study by Shumway *et al*. (1964), these differences may be related to the more extreme and prolonged nature of oxygen depletion. However, Youngson *et al*. (2004) only took measurements of oxygen content every two weeks, so it is not possible to definitively draw the same conclusion. The absence of a difference in total mass among treatments in the present study is probably related to lower yolk sac absorption and thereby higher contribution of the yolk sac to total body mass of hypoxic individuals.

In agreement with other studies (Hamor & Garside 1977; Roussel 2007), all hypoxic treatments gave rise to alevin that had a lower body mass as a proportion of total mass. This effect was most pronounced in the late sag treatment. This is attributed to the fact that yolk-feeding fish such as Atlantic salmon distribute energy between growth and metabolism. As oxygen supply is reduced, there is a shift towards less efficient anaerobic processes (Kamler 2008). This means that the rate of conversion of yolk sac tissue into body tissue is reduced.

The use of caudal fin rays as an indicator of alevin development at hatch enabled observation of the extent of developmental retardation and demonstrated that, in all cases, hypoxia during incubation gave rise to underdeveloped alevin. Observation of the raw data suggests that the extent of developmental delay was strongest in the embryos exposed to the late sags treatment. However, using formulae developed by Gorodilov (1996), it was possible to show that the degree of developmental delay was remarkably similar across all sag treatments. This suggests that the primary cause of the difference in developmental state between the alevin of the late sag treatment and the early and median sag treatments was related to variance in hatch timing as opposed to a differential response to hypoxia at different developmental stages. This finding is important as it demonstrates that oxygen sags such as those caused by groundwater intrusion can impede development of Atlantic salmon embryos.

Implications for survival

This study shows that Atlantic salmon embryos experiencing hypoxia during incubation are likely to hatch at an unfavourable time in a sub-optimal condition. Aggregation of hatch timing is an adaptive measure to limit the impacts of predation (Pulliam & Caraco 1984) through appetite satiation or exceeding handling capacity of the predator (Begon & Mortimer 1986). Deviation from the time of peak hatch in either direction observed here dilutes this effect and increases the chances of an individual encountering a predator such as the bullhead (C*ottus gobio*, Roussel 2007) or burbot (*Lota lota*, Louhi *et al*. 2011). In addition, the smaller size and greater yolk sac mass of Atlantic salmon embryos exposed to the late sags treatment reduces their mobility, so inhibits their escape response (Parker 1971; Fresh & Schroder 1987; Sogard 1997). Further, hypoxia during incubation reduces the number and size of white muscle fibres (Matschak *et al*. 1997), which are essential for rapid acceleration and high swimming speeds (Valente *et al*. 1999), and thereby escape from predators.

Salmonid embryos exposed to stressors such as weathered crude oil during incubation sometimes display delayed mortality and retarded growth into much later life-stages (Heintz *et al*. 2000). No such delayed mortality has been observed for salmonid eggs experiencing hypoxia (Seager *et al*. 2000; Geist *et al*. 2006; Roussel 2007). However, studies over a period of 2-3 years or more would help to inform the long-term impacts of Atlantic salmon eggs exposed to hypoxia.

A second critical phase in the juvenile life-stages is the completion of yolk sac absorption and emergence of fry from the gravels. A major weakness of this study is that it was not possible to determine whether the hypoxia-induced developmental lag at hatch observed in all the sag treatments would have resulted in delayed emergence. However, other salmonids that hatch later than conspecifics as a result of hypoxic incubation conditions are also likely to emerge from the gravels later (Carlson & Siefert 1974; Roussel 2007). The time

433 of emergence represents a second period of vulnerability in which mortality rates, due to
434 predation from species such as brown trout, can be high (Einum & Fleming 2000). Therefore,
435 late entry into this stage reduces the prey dilution effect. In addition, fry become territorial at
436 this stage, so a developmental lag would mean juveniles emerge at a time of limited habitat
437 availability. If a juvenile is unable to find a territory it can suffer mortality through starvation
438 or predation (Einum & Fleming 2000; Einum & Nislow 2005). The fact that a large
439 proportion of mortality can occur in the fry stage demonstrates the need to study the impacts
440 of groundwater intrusion into later life-stages than are presented here.

441 The impact of variable hatch timing and reduced alevin fitness could have negative
442 implications for future populations. The global decline of Atlantic salmon (ICES 2015)
443 means that, in many locations, populations will be lower than the carrying capacity of the
444 natal stream. In these instances, low post-hatch density leads to under-utilisation of resources
445 (Aprahamian *et al*. 2003), so population size can be limited by abiotic conditions. Therefore,
446 reduced post-hatch fitness as a result of hypoxia during incubation could limit population
447 abundance (Sinclair 1989; Jonsson *et al*. 1998).

448 The likelihood of embryos being exposed to hypoxic groundwater during incubation
449 is strongly influenced by spawning site selection of the maternal fish. Paradoxically,
450 geomorphological features such as knickpoints and channel confinement, which provide ideal
451 spawning substrates, are likely to induce intrusion of groundwater into spawning gravels
452 (Baxter & Hauer 2000; Malcolm *et al*. 2005). The high spawning density often observed in
453 these locations (Malcolm *et al*. 2005) means that a substantial number of eggs are likely to be
454 exposed to hypoxic groundwater during incubation. However, this effect would be dampened
455 if maternal fish have the ability to identify locations susceptible to groundwater input.
456 Surprisingly, some maternal Pacific salmonids exhibited a preference for sites susceptible to
457 groundwater input (Garrett *et al*. 1998; Baxter & McPhail 1999). However, it is important to

note these sites were dominated by short-residence groundwater that were not significantly oxygen depleted. Whether female Atlantic salmon have the ability to detect areas of hypoxic groundwater upwelling requires greater understanding. It is important to note that the observed 'risk-spreading', whereby male and female Atlantic salmon distribute their gametes across multiple redds (Taggart *et al*. 2001; Youngson *et al*. 2004) potentially limits the cumulative impact of groundwater on embryonic survival.

Variability between families could result in differences in terms of the consequences of groundwater upwelling on Atlantic salmon post hatch fitness. Eggs of different families of Chinook salmon (*Oncorhynchus tshawytscha*) have been shown to respond differently to thermal variability (Steel *et al*. 2012) and there is a distinct difference between the eggs of different families in terms of natural survival rates (Johnson *et al*. 2012; Roni *et al*. 2015). This suggests an important genetic influence on incubation success and that oxygen stress could affect eggs of different families unequally.

Implications for river management

Effective management of factors that influence groundwater-surface water interactions is important to limit effects on incubating salmonids. Although groundwater upwelling is a natural process, anthropogenic activity can increase its frequency and severity. For example, the introduction of log steps to reduce erosion and scour can enhance groundwater upwelling (Schindler Wildhaber *et al*. 2014). River abstraction will reduce surface water input into the hyporheic zone (Hancock 2002) and dredging could deepen the incubation zone relative to the hyporheic zone, increasing the influence of groundwater. In addition, climate change predictions suggest an increase in precipitation across much of the European range of Atlantic salmon (Kovats *et al*. 2014). As groundwater intrusion is most intense following periods of prolonged rainfall and subsequent water table elevation, these predictions mean that groundwater upwelling is likely to become more frequent during the Atlantic salmon incubation period (Jackson *et al*. 2011).

Other anthropogenic activities could also reduce groundwater quality. For example, the introduction of organic fertilisers into the ground can strip oxygen from water (Hancock 2002). This intensifies the naturally occurring reduction of oxygen in groundwater. Further, human activities such as mining (Hancock 2002) can pollute groundwater which, combined with low oxygen levels, could further reduce Atlantic salmon incubation success (Heugens *et al*. 2001).

This study has shown that low oxygen regimes similar to those caused by upwelling groundwater can reduce the post-hatch fitness of Atlantic salmon. Initial observations imply that this impact is strongest when hypoxia occurs at the latest stages of egg development but more detailed analysis showed that one of the main driving factors behind reduced fitness at hatch is deviation in hatch timing. It is likely that underdeveloped alevin hatching at a suboptimal time will have poor rates of survival due to predation. This impact will be

enhanced at later life-stages if the developmental delays continue. However, these longer-term impacts require further investigation. The findings of the present study demonstrate the need for a better understanding of the natural and anthropogenic controls on low dissolved oxygen groundwater upwelling.

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Tables

Table 1. Target oxygen concentrations of all oxygen treatments of the present study.

Continuous Treatments		Sag Treatments							
Control	Chronic			Early Sags		Median Sags		Late Sags	
Oxygen Saturation (%)	Oxygen Saturation (%)	Pulse number	Oxygen Saturation (%)	Start (DD)	End (DD)	Start (DD)	End (DD)	Start (DD)	End (DD)
Maximum	60	**1**	**50**	134	142	229	236	317	324
		2	**50**	152	161	244	252	331	338
		3	**50**	170	178	259	267	345	352
		4	**30**	185	192	275	284	358	364
		5	**30**	198	204	293	300	371	379
		6	**30**	209	214	309	317	387	395
		7	**10**	221	229	324	331	403	410
		8	**10**	236	243	338	345	418	426
		9	**10**	252	259	351	358	435	441
		10	**10**	267	275	364	371	448	456

Note. Each treatment was applied to a different group of 300 eggs of each maternal fish. Eggs of each maternal fish were subjected to all of the treatments described. Eggs of the continuous treatments were exposed to stated oxygen concentrations throughout experiment. For sag treatments, each pulse lasted for 24hrs and was separated by a period of oxygen recharge, also of 24hrs. The time of each oxygen pulse is given in degree days to aid identification of developmental states at which embryos were most sensitive to low oxygen levels.

Table 2. Incidence of hatching and death by treatment (N=6,000)

Treatment	N	Total hatched	Total deaths	Time exposed to risk (1 000DD)	Hatching rate from 350DD (per 1 000DD)		
					Est.	95 % CI lw bd	95 % CI up bd
Control	1,200	1 141	59	557.382	8.09	7.634	8.574
Chronic	1,200	1 123	77	560.527	7.78	7.342	8.253
Early sags	1,200	1 149	51	578.538	7.12	6.717	7.54
Median sags	1,200	1 151	49	573.316	7.32	6.905	7.751
Late sags	1,200	1 022	178	536.204	8.47	7.968	9.008

Note. Hatching rate is taken from 350DDs to increase data resolution.

Table 3. Comparison of the incidence of hatching among oxygen treatments.

Treatment	Control	Chronic	Early Sags	Median Sags	Late Sags
Control	-	0.493	0.001	0.017	0.02
Chronic	0.972	-	0.011	0.091	0.003
Early Sags	0.874	0.899	-	0.391	0.001
Median Sags	0.905	0.932	1.036	-	<0.001
Late Sags	1.104	1.136	1.264	1.219	-

Note. Lower half of matrices indicate the incidence of hatching ratio of the row treatment group over the column treatment group (i.e. a value >1 indicates row treatment has higher incidence of hatch than column treatment). Corresponding p-values produced using a log-rank test of significance are reported in the top half.

Table 4. Details of alevin biometrics separated by treatment. Error values indicate standard deviation.

Treatment	**Control**	**Chronic**	**Early Sags**	**Median Sags**	**Late Sags**	**Total**
Length (mm)	17.22 ± 0.83	17.34 ± 0.81	17.19 ± 0.85	17.24 ± 0.81	16.18 ± 1.00	17.05 ± 0.99
Total dry mass (mg)	48.16 ± 9.02	48.77 ± 8.53	47.00 ± 8.67	47.36 ± 8.77	50.03 ± 8.32	48.17 ± 8.71
Dry body mass proportion (%)	10.94 ± 2.59	9.58 ± 2.11	10.25 ± 2.41	10.07 ± 2.50	7.50 ± 1.90	9.81 ± 2.58
Caudal fin rays	14.42 ± 0.90	13.23 ± 1.06	12.87 ± 1.33	12.44 ± 0.77	8.95 ± 0.62	12.80 ± 1.88
Estimated number of Caudal Fin Rays at 526DDs	15.17 ± 1.02	13.98 ± 1.14	12.96 ± 1.36	12.64 ± 0.79	12.79 ± 0.94	13.52 ± 1.10

Figure Captions

Figure 1. Small inset map shows global range of Atlantic salmon in maroon (Taken from FAO 2016). Larger map of mainland United Kingdom shows rivers with native Atlantic salmon populations in blue. Locations from which eggs were sourced for this experiment are shown in red.

Figure 2. Systematic diagram of recirculating system at the research facility. Arrows indicate direction of water flow. Location of each treatment is labelled. Numbers within egg chambers indicate location of eggs of each maternal fish: 1 = River Ure; 2 = Farmed; 3 = South Tyne; 4 = River Rede.

Figure 3. Estimated oxygen regimes Treatment: (A) Control; (B) Chronic; (C) Early sags; (D) Median sags; (E) Late sags. Values calculated using equation 1. Scatter points represent daily spot check values.

Figure 4. Image taken under a Nikon E100 microscope at 100x magnification of the caudal fin of a newly hatched alevin. Visible are caudal fin rays that were used to estimate alevin developmental state.

Figure 5. Kaplan-Meier estimate of the hatching function by treatment (N=6 000). Vertical marks on curves indicate incidence of mortality. For ease of identification, curves are labelled A-E to match the corresponding treatment.

Figures

Figure 1

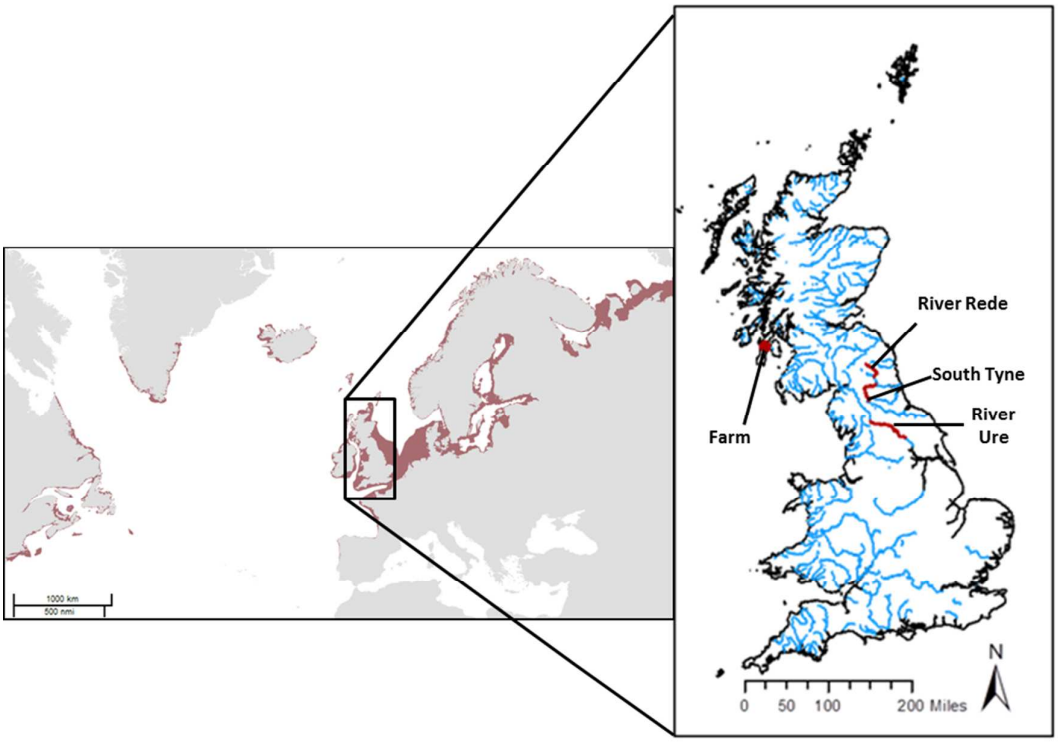

Figure 1. Small inset map shows global range of Atlantic salmon in maroon (Taken from FAO 2016). Larger map of mainland United Kingdom shows rivers with native Atlantic salmon populations in blue. Locations from which eggs were sourced are labelled and are in heavier red lines.

Figure 2

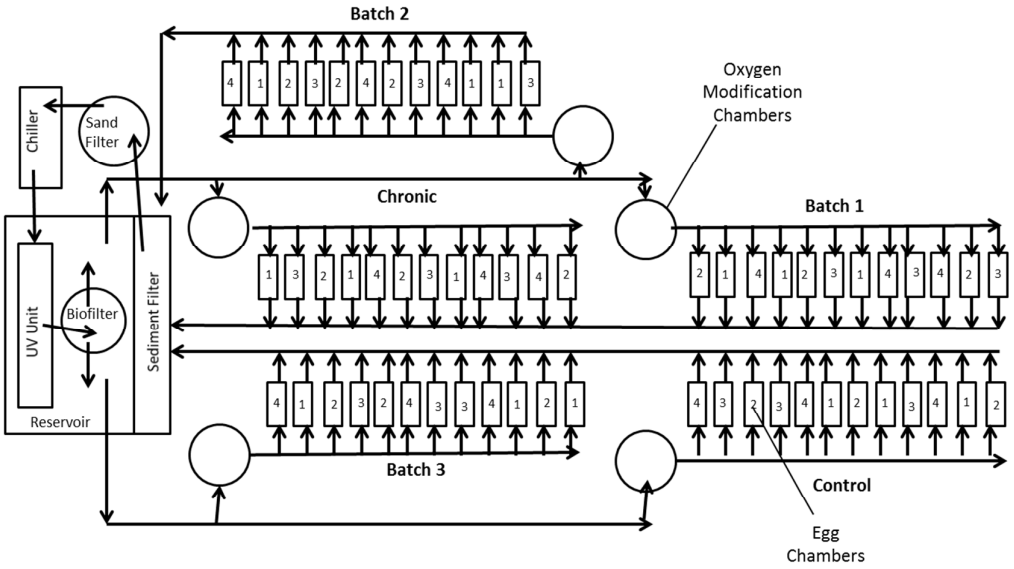

Figure 2. Systematic diagram of recirculating system at the research facility. Arrows indicate direction of water flow. Location of each treatment is labelled. Numbers within egg chambers indicate location of eggs of each maternal fish: 1 = River Ure; 2 = Farmed; 3 = South Tyne; 4 = River Rede.

Figure 3.

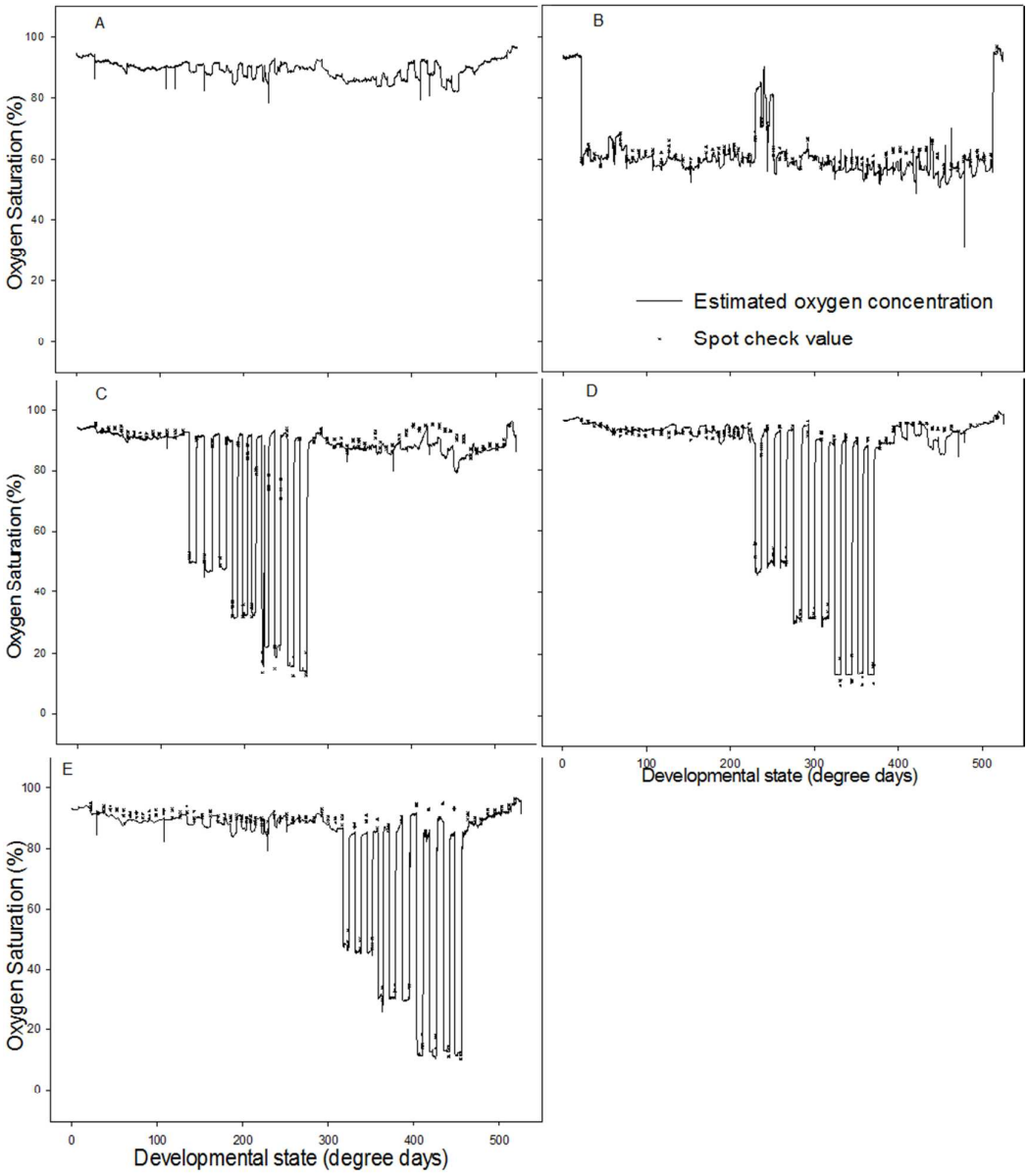

Figure 3. Estimated oxygen regimes Treatment: (A) Control; (B) Chronic; (C) Early sags; (D) Median sags; (E) Late sags. Values calculated using equation 1. Scatter points represent daily spot check values.

Figure 4.

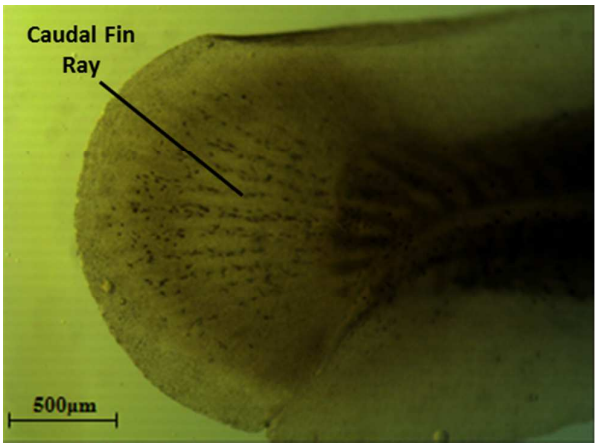

Figure 4. Image taken under a Nikon E100 microscope at 100x magnification of the caudal fin of a newly hatched alevin. A caudal fin ray that was used to estimate alevin developmental state is labelled.

Figure 5.

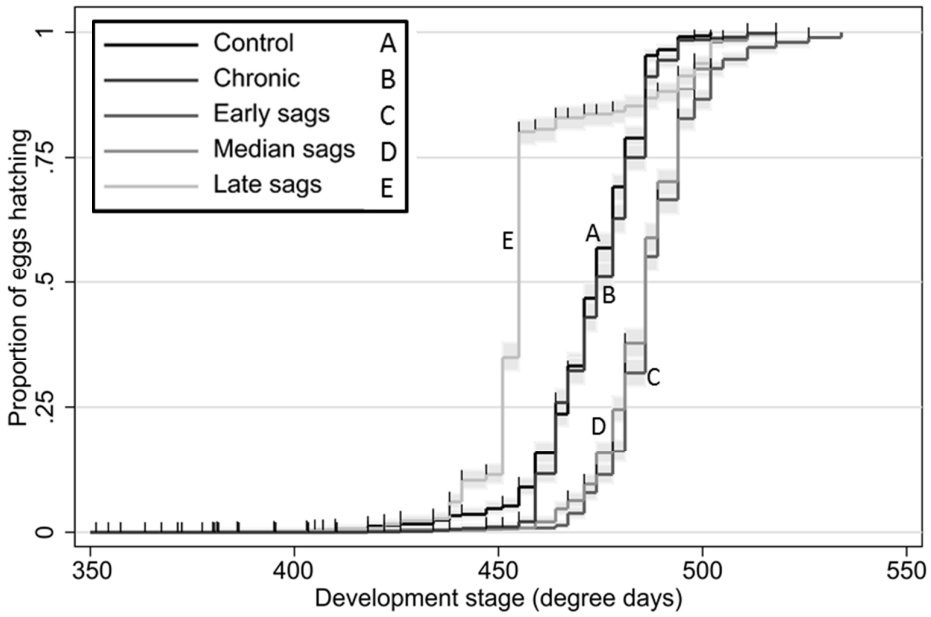

Figure 5. Kaplan-Meier estimate of the hatching function by treatment (N=6 000). Vertical marks on curves indicate incidence of mortality. For ease of identification, curves are labelled A-E to match the corresponding treatment.