The role of physical structure and micronutrient provisioning in determining egg quality and performance in fish

By

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"*I have not failed. I've just found 10,000 ways that won't work*"

Thomas A. Edison

Abstract

This thesis examined novel and previously utilised parameters of egg quality to determine and define reproductive success in farmed and wild salmonids. The effect of holding environment and inter-female variation on salmonid egg quality was also examined. Furthermore, two nutritional feed trials were undertaken to investigate whether organic Se, supplemented into salmonids broodstock diets, was vertically transferred to their eggs and what affect this dietary supplementation had on egg quality. Finally, the possibility that morphological and biochemical adaptations are present on the chorion of eggs from European whitefish (*Coregonus lavaretus*) was investigated in a resident Scottish population.

Chapter 2 examined methods to determine egg quality using eggs from a single population of brown trout (*Salmo trutta*). Egg survival, provided a biologically relevant definition for egg quality, which was used throughout this study to assess the importance of selected egg quality parameters. Based on a review of the literature and the relationship between parameters of egg quality and egg survival rates, three determinants of egg quality were chosen for further examination. These were chorion breaking strength, elemental concentrations within the egg and the protein profile of the chorion.

Brown trout broodstock from a single population were separated prior to spawning and exposed to two different holding units, 'Ae system' or 'S.C.E.N.E. system' at two sites. Eggs were stripped from females and 13 determinants of egg quality collected, analysed individually, combined by principle components analysis into an integrated egg quality score which was validated against egg survival. The multivariate egg quality score differed significantly between fish held in the Ae and S.C.E.N.E. systems. Egg survival, chorion breaking strength and Se chorion concentrations were higher in eggs produced by broodstock held in the S.C.E.N.E. system compared to those in the Ae system. Alternatively, chorion concentrations of P and K were higher in eggs from fish held in the raceway system. This data highlights the complex interactions between the holding environment and pre-ovulating fish and resultant egg quality.

The variation in egg survival in individual Atlantic salmon (*Salmo salar*) reared in the same environment was assessed and used to examine the suitability of chorion measurements as parameters of egg quality. There was a significant difference in the egg survival rates between individual salmon. Results also show that there was also variation in egg survival, chorion breaking strength, chorion elemental concentrations and chorion protein concentrations and profiles between individual Atlantic salmon. Subsequent analysis of the data showed that there was no difference in these egg quality parameters between high and low egg survival rates. Furthermore, there was no correlation between egg survival and the chorion quality parameters recorded during this study. The results show that individual variation between fish is an important factor affecting egg quality.

Broodstock Atlantic salmon were fed a standard commercial diet, with or without the addition of a supplemented nutritional mix, which included 0.5mg/kg of Sel-plex (organic Se). The Se content of the eggs and livers of each fish were assessed as was egg survival rates and proteomic analysis of the egg chorion. Concentrations of Se in the eggs of the individuals fed the supplemented diet were significantly higher than those fed the non-supplemented diet. However, the egg survival rate was also significantly lower in the supplemented group of fish. The assessment of the chorion protein profile and its proteomic structure was inconclusive. These results support the hypothesis that dietary selenium is vertically transferred to immature eggs during oocyte development. The lack of a linear relationship between Se egg concentrations and egg survival suggests that the lower survival rates of eggs from broodstock fed the supplemented diet in this trial was due to another nutritional component of the diet rather than the Se.

Selenium enriched eggs from Atlantic salmon fed a supplemented diet and eggs from conspecifics fed a non-supplemented diet were tested for their ability to resist infection by *Saprolegnia* under incubation conditions similar to those used by the aquaculture industry. There was no significant difference in the presence/absence of infection, infection rate or survival between eggs produced by Atlantic salmon fed the supplemented and non-supplemented diet. Therefore, it was concluded that supplementation of broodstock diet does not alter the resistance of eggs to *Saprolegnia*.

The presence of adhesive mechanisms on the surface of European whitefish eggs was examined from a population found within Loch Eck, Scotland. European whitefish eggs remain non-adhesive in a solution chemically similar to ovarian fluid, but become adhesive seconds after contact with water. Examination of the ultrastructure of the chorion showed that the morphology altered significantly after contact with water with nodule-like protuberances attached to connective filaments on the surface, present in water hardened but not non-water hardened eggs. Biochemical analysis also showed the presence of Chain A, RNase ZF-3e in the chorion of water hardened but not non water hardened eggs. Histochemical staining of the chorion showed that the externa, but not the interna stained positively for the presence of glycoproteins. Egg adhesive mechanisms allow European whitefish eggs to remain in optimal spawning grounds where factors such as mechanical damage, predation, desiccation and hypoxia are minimised.

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Dedication

This thesis is dedicated to my mother, Glenda Mary Murray and in loving memory of my father Andrew David Murray, when people told me I wouldn’t be able to do it, you both told me I could, then made me believe it.

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Author's declaration

The material presented in this thesis is the result of research conducted between October 2008 and October 2011, under the supervision of Prof. Colin Adams, Dr. Maureen Bain and Prof. Sally Solomon. This work has not been submitted as part of any other degree and is based on individual research carried out by myself. Any published or un-published material not of my own has been acknowledged in the text

David Murray

1 General Introduction

1.1 The global impact of the aquaculture industry

Aquaculture of fish, shrimp, shellfish and seaweed has been a significant source of protein for humans for nearly 4,000 years (Iwama 1991). When first developed, fish farming was practiced by individuals in order to provide food for their families, however due to the growth of the human population and the subsequent increase in dietary protein requirements, this initially subsistence based practice grew into a worldwide food production industry. With the benefit of intense research concerning the biological, nutritional and behavioural needs of wild fish under farmed conditions, the worldwide aquaculture production has grown dramatically (Hall *et al.*, 2011). For example, by 2000 the aquaculture industry provided 22% of global fish production resulting in a new level of importance in both the modern food supply and as a source of employment (Matthews and Hammond, 1999). On average world aquaculture production has grown at an annual rate of 8.4% since 1970 and reached 65.8 million tonnes in 2008, however conservative estimates indicate that in 2010 this number had increased to approximately 100 million tonnes (Hall *et al.*, 2011; Matthews and Hammond, 1999).

China supplies 61.5% of global aquaculture production with a further 29.5% supplied by the rest of Asia, 3.6% from Europe, 2.2% from South America, 1.5% from North America, 1.4% from Africa and 0.3% from Oceania (Hall *et al.*, 2011). In both China and Asia aquaculture production is dominated by cyprinids, but particularly by 4 species of carp, silver (*Hypophthalmichthys molitrix*), grass (*Ctenopharyngodon idella*), common (*Cyprinus carpio*) and bighead (*Hypophthalmichthys nobilis*) (Hall *et al.*, 2011). In contrast Europe and South America aquaculture is predominately based on the production of salmonids, including but not limited to Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) (NACA/FAO, 2001). In North America, aquaculture production is broadly spread across a variety of classes and families including malacostracans, bivalves, salmonids, ictalurids, cyprinids and cichlids (Hall *et al.*, 2011). African aquaculture production is almost exclusively for tilapia, while in Oceania the industry is focused on shrimp and prawn production (Hall *et al.*, 2011).

1.2 Fish egg quality

Both farmed and wild fish are required to produce good quality eggs in order to maintain their populations. However, variation in egg quality, defined as the ability of an egg to be fertilised, reach key embryonic developmental stages and hatch, has been identified as one of the main factors limiting the expansion of both marine and freshwater aquaculture species. The issue of variation in egg quality has also been implicated as the cause of fluctuations in recruitment observed in many wild fish stocks (Bobe and Labbe, 2010; Brooks *et al.*, 1997; Kjorsvik *et al.*, 1990).

There are a number of obstacles to studying egg quality in fish. One of the major problems is that previous studies have used a number of different definitions for egg quality, including but not limited to egg survival rates and the time taken to reach key embryonic stages of development (Bobe and Labbe, 2010; Brooks *et al.*, 1997; Kjorsvik *et al.*, 1990). There are further problems with establishing parameters of egg quality that provide reliable predictions for performance, as often the reliability of these parameters varies between species, environments and studies. Within the aquaculture industry good quality eggs are defined as those exhibiting low mortalities at fertilisation, eyed stage, hatching and first feeding, thus, for aquaculture at least, the term egg quality has become synonymous with egg survival. While the ability of an egg to successfully fertilise, maintain an embryo and hatch may be considered the ultimate measure of egg quality, it fails to describe the factors which allowed the egg to proceed through each of these developmental stages (Brooks *et al.*, 1997). Therefore, from a biological standpoint the quality of an egg must be defined by, and dependent on, the intrinsic properties of the egg itself. For example, the mRNA transcripts present prior to fertilisation provide the egg with proteins, including those associated with membrane and cytoskeleton formation (Tata 1986).

The aquaculture industry requires measures of egg quality based on mechanisms that support successful development and are able to accurately identify egg quality issues during the earliest stages of embryonic development (Kjorsvik *et al.*, 1990). Early studies therefore focused on the size and appearance of unfertilised eggs and attempted to use these criteria to tentatively estimate the developmental potential of the egg pre and post fertilisation (Bobe and Labbe,

2010; Bromage *et al.*, 1992). However, despite large variations in the weight of eggs produced by individual fish, Bromage *et al.*, (1992) found that in salmonids held under normal aquaculture conditions, larger eggs produced similar survival rates compared to smaller eggs. Hatcheries incubating eggs from marine fish distinguished between good and bad quality by using the egg's ability to float or sink in seawater (Mcevoy 1984; Carrillo *et al.*, 1989; Kjorsvik *et al.*, 1990). While this measure appears to work for the majority of marine species which utilise pelagic spawning behaviour, the positive relationship between buoyancy and egg quality is not consistent in many other marine fish, including the commercially important Atlantic halibut (*Hippoglossus hippoglossus*) (Bromage *et al.*, 1994).

Due to the uncertainty concerning the biological relevance of these measurements as determinants of egg quality, other physiological parameters have been investigated. For example, several authors have suggested that egg quality, as defined by the ability of an egg to reach key embryonic phases, can be predicted based on the physio-chemical parameters of the ovarian fluid, in which the unfertilised eggs are bathed after release from the ovary (Aegerter and Jalabert, 2004; Flauvel *et al.*, 1993; Lahnsteiner, 2000). Low pH values observed in the ovarian fluid of both marine and freshwater fish species has been associated with a reduction in egg quality (Bobe and Labbe, 2010). Subsequent examination of this occurrence found that the drop in ovarian fluid pH was caused by egg yolk proteins contaminating ovarian fluid during a process referred to as post ovulatory ageing (Rime *et al.*, 2004). This process occurs when ovulated eggs, freshly released from the ovary are retained in the coelomic cavity for a prolonged period of time. Despite the success of these studies linking egg quality to the physio-chemical parameters of the ovarian fluid, it should be noted that the variation in the mean pH values during post ovulatory ageing was limited and that there was no significant linear relationship between ovarian pH and egg survival rates (Aegerter and Jalabert, 2004; Lahnsteiner 2000).

Further studies have examined a number of egg variables such as mRNA transcription factors, lipid droplet assessment, enzyme activity within the yolk and chorion permeability in order to ascertain parameters of egg quality (Bobe and Labbe, 2010; Brooks *et al.*, 1997; Kjorsvik *et al.*, 1990; Lahnsteiner *et al.*, 1999). The use of such estimators is limited under normal hatchery conditions

and a lack of a consistent relationship between these measurements and egg quality, as defined by the egg's ability to be fertilised, survive up to specific embryonic developmental stages and then hatch, continues to hamper research efforts to improve the reproductive potential of cultured fish species. In addition, information regarding the relationship between egg quality and components of the egg, such as the chorion remains limited, despite the importance of such structures to egg survival.

1.3 The sequential construction of the fish egg.

In teleost fish, an egg is the final product of oocyte development and once ovulated takes up very little if any nutrients, therefore, all contents of the egg must be incorporated into the oocyte while it is still attached to the ovary (Brooks *et al.*, 1997). This situation is very different from that of eutherian mammals, where nutrients within the egg are needed only to initiate embryonic development; once the egg attaches to the uterine wall, all other nutrients needed for development are provided by the female (Brooks *et al.*, 1997). The eggs of oviparous (egg laying) animals are thus understandably larger than in mammals, for example fish eggs measuring 1mm in diameter are 23,000 times larger, by volume than a human egg, and the eggs of the coelacanth (*Latimeria chalumnae*) are more than a million times larger in volume than a human egg (Brooks *et al.*, 1997).

In all teleost species, immature egg cells, called oocytes, undergo the same basic pattern of development, regardless of their reproductive strategy. The major developmental events occurring during oocyte formation can be broadly classified into six stages; these are oogenesis, primary oocyte growth, cortical alveolus stage, vitellogenesis, maturation and ovulation (Tyler and Sumpter, 1996). A summary of these stages are described as follows.

1.3.1 Oogenesis

In all vertebrates oocytes are derived from non-dividing primordial germ cells during the early stages of embryogenesis and proliferation of these germ cells gives rise to a stem cell population of oogonia (Brooks *et al.*, 1997; Tyler and Sumpter, 1996). In other vertebrates oogonia can subsequently enter oogenesis,

forming oocytes, or degenerate before the adult female reaches sexual maturity. In teleosts, however dividing oogonia persist in the ovary and continue to divide by meiosis to form oocytes.

1.3.2 Primary oocyte growth

The initial stages of primary oocyte growth are characterised by a period of intense RNA synthesis followed by an increase in the volume of the oocytes. This increase in growth is caused by the formation of the Balbiani body, a membrane-less transitory structure with a heterogeneous content of cytoplasmic organelles, such as mitochondria, multivescular bodies, endoplasmic reticulum and Golgi elements, together with fibrogranular material (Tyler and Sumpter, 1996). During this stage, an acellular envelope develops around the oocyte which will continue to differentiate and increase in complexity throughout oocyte development, eventually forming the tough eggshell known as the chorion (Brooks *et al.*, 1997).

1.3.3 Cortical alveolus stage

Cortical alveoli are the first cytoplasmic structures within the oocyte which can be viewed using light microscopy (Tyler and Sumpter, 1996). In the majority of teleosts these structures contain a polysialoglycoprotein which is thought to be synthesised endogenously by the female and transferred to the developing oocyte (Inoue and Inoue, 1987). Towards the end of this stage, the cortical alveoli almost fill the entire oocyte cytoplasm, however during vitellogenesis they progress to the periphery of the oocyte and during the activation phase of egg development they release their contents into the perivitelline space (Tyler and Sumpter, 1996). Lipid droplets often appear in oocytes during the cortical alveolus stage and may continue to gather during the remaining growth phases.

1.3.4 Vitellogenesis

Vitellogenesis is the principle event responsible for the large growth of oocytes during development and can account for as much as 95% of the final egg size (Tyler *et al.*, 1991). In most salmonids, oocytes can remain in the vitellogenic growth phase for 9 months or more (Tyler and Sumpter, 1996). During this time the circulating concentrations of the hepatically derived plasma precursor,

vitellogenin (VTG) in the blood increases and when it reaches the ovary is sequestered, processed and packaged into oocytes by receptor mediated endocytosis (Tyler and Lancaster, 1993). During this phase VTG is proteolytically processed into smaller yolk proteins which accumulate in fluid filled spheres or globules, these molecules are responsible for the enormous growth rates observed in oocytes (Tyler *et al.*, 1991). These proteins are also responsible for energy and building substrates for early embryonic development (Arukwe and Goksoyr, 2003)

1.3.5 Maturation and ovulation

During the previous phases, the oocyte germinal vesicle, a large nucleus precursor, is arrested in meiotic prophase, however when meiosis resumes, via a hormonal signal, this causes the germinal vesicle to breakdown and the chromosomes to enter first meiotic metaphase (Brooks *et al.*, 1997). Most evidence indicates that protein uptake in oocytes continues during the maturation phase but stops abruptly when the germinal vesicle is broken down (Wallace and Selmen, 1985). The breakdown of the nucleus precursor not only causes the oocyte to stop sequestering maternally derived materials it also causes the egg to become relatively impermeable (Tyler and Sumpter, 1996). The oocyte is then released from the ovary into the body cavity where it is ready for fertilisation (Brooks *et al.*, 1997).

1.4 Key stages of embryonic development post-ovulation

The eggs of oviparous fish are ejected from the body cavity into the external environment where they will be fertilised by male gametes. The micropyle and nucleated blastodisc are located in a specialised region of the egg referred to as the animal pole. The function of these structures is to ensure the successful fertilisation of eggs by spermatozoa, the male gametes (Kobayashi and Yamamoto, 1981). Successful fertilisation requires a spermatozoon to successfully enter the micropylar canal and fuse with the egg membrane. If the egg remains un-fertilised when it makes contact with water it produces a chain reaction which will result in the egg ‘hardening’ (Ballard 1973). Alternatively if the egg is fertilised when it makes contact with the water then this causes the egg to ‘activate’ (Ballard 1973). The cause of egg activation and/or hardening

has been identified as the transient rise in intracellular free calcium, due to the physiochemical differences between the internal and external environment (Gilkey 1983; Gilkey *et al.*, 1978; Coward *et al.*, 2002). This process causes a complex series of physiological and morphological changes to the egg including water being osmotically drawn across the chorion into the egg causing the vitelline membrane to detach from the inner surface of the chorion and forming the perivitelline space, the cross polymerisation of proteins in the chorion causing this structure to harden, the closure or narrowing of the micropyle, metabolic stimulation, and if the egg has been successfully fertilised, the continuation of meiosis and embryogenesis (Bement 1992). After fertilisation and activation the very basic structure of the egg contains 6 main structural elements; the micropyle, nucleated blastodisc, perivitelline space, vitelline membrane, chorion and yolk (Figure 1-1).

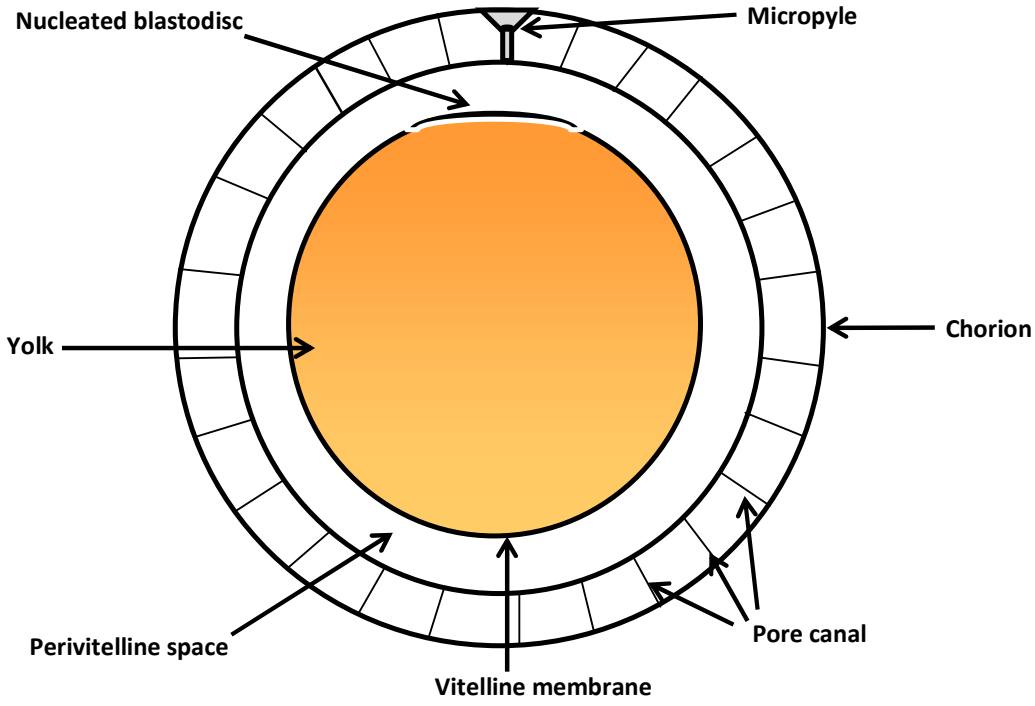

Figure 1-1: The general structure of a fertilised and activated salmonids egg

The 'yolk' filled centre of the egg contains all the vital materials necessary to form the embryo and continue its development within the egg. Most of the ribosomes, DNA and RNA polymerases, histone proteins, transcription and translation factors and proteins needed for embryonic development are synthesised within the developing oocyte (Tata 1986). Located towards the

centre of the egg, enzymes such as cathepsin degrades VTG into yolk proteins for storage and also regulates the degradation of these proteins into free amino acids for use by the developing embryo (Sire *et al.*, 1994).The amino acids, phosphates, lipid and calcium required to form the embryo are obtained from the large complex VTG molecule. This is also responsible for the major yolk proteins, lipovitellin and phosphoprotein (Specker and Sullivan, 1994). Additional lipids are however also sequestered within the yolk from the circulation during development, providing an additional source of energy for the developing embryo (Brooks *et al.*, 1997). Although yolk proteins and lipids represent the majority of material within the ovulated oocyte, other molecules such as vitamins and minerals are present in far lower quantities but are equally important in producing viable offspring (Brooks *et al.*, 1997). For example, some vitamins and minerals are required for enzyme activities, including hatching (Brown and Lynam, 1981). Knowledge concerning the hormonal content of fish oocytes is limited, however some studies have reported the presence of thyroid hormones, cortisol and several sex steroids within ovulated eggs (Babin 1992; Bobe and Labbe, 2010; Cyr and Eales, 1992).

The development of the embryo itself involves 3 main stages; cleavage (cell division), epiboly (tissue formation) and organogenesis (Gilbert 2000; Kjorsvik *et al.*, 2004) (Figure 1-2). The time the egg takes to complete these stages is dependent on a number of environmental parameters, such as temperature, but also the species being investigated. For example, if incubated in optimal conditions, the zebrafish (*Danio rerio*) egg undergoes cleavage, epiboly, organogenesis and hatches approximately 72 hours after fertilisation (Kimmel *et al.*, 1995). In comparison, under optimal conditions Atlantic salmon eggs proceeds through each of these embryonic development phases and hatches approximately 3-4 months after fertilisation.

In the fertilised egg of all teleost species, cell division begins with the first cleavage of the nucleated blastodisc to form two cells, which continue to divide until the number of cells can no longer be counted (around 32 cell divisions) after which the cells take a granulated appearance and the embryo can be clearly detected (Gilbert 2000; Kjorsvik *et al.*, 2004; Pittman *et al.*, 1990). The second stage (epiboly) involves the cells formed during the cleavage phase beginning to specialise to form different tissues (Gilbert 2000; Kjorsvik *et al.*,

2004). The blastodisc expands over the surface of the yolk eventually forming the yolk sac and body cavity, by the time a third of the surface of the yolk has been covered, the head, somites, muscle tissue, spinal cord and optic vesicles can be detected (Gilbert 2000; Kjorsvik *et al.*, 2004). Within the aquaculture industry the end of the epiboly stage is synonymous with the 'eyed' stage of development, whereby the eyes have fully developed and can be easily detected without the aid of a microscope (Gilbert 2000; Kageyama 1980; Kjorsvik *et al.*, 2004). The final phase (organogenesis) begins with the formation of the fins, followed by the development of the circulatory system and external morphological features such as the operculum and gills (Falk-Petersen 2005; Gilbert 2000; Kjorsvik *et al.*, 2004). At the end of organogenesis the body of the embryo has fully developed and is pigmented, ready for hatching (Kjorsvik *et al.*, 2004).

Knowledge of the mechanisms underlying the processes of oocyte maturation and how these processes are coordinated is essential for understanding how different factors can influence fish reproductive status. However, the formation of mature eggs and subsequent embryonic development of the fertilised egg are very complex processes and despite its importance, knowledge of the coordinated assembly of the oocyte is far from complete (Brooks *et al.*, 1997).

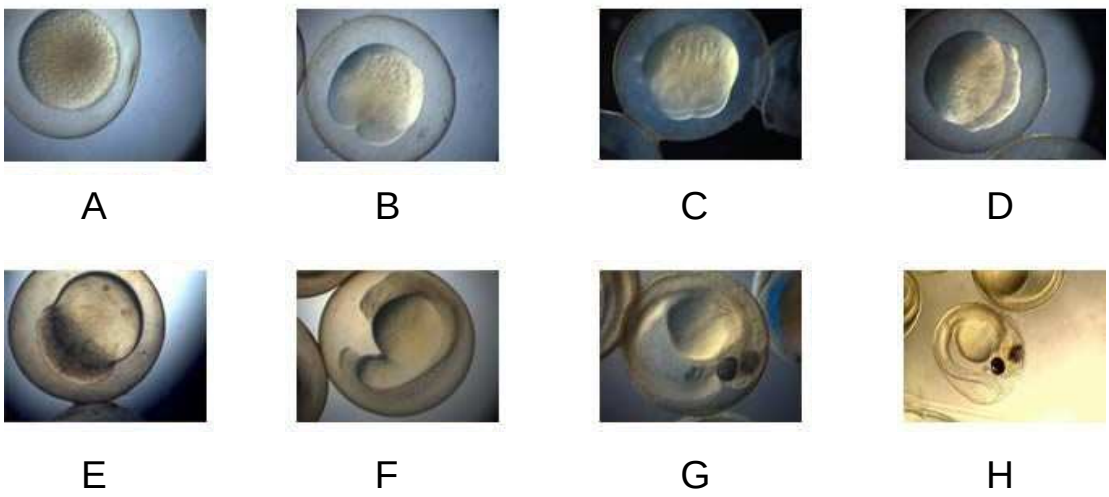

Figure 1-2: Embryonic development of a fish egg. A-D) Cell cleavage of the nucleated blastodisc. E) End of cell cleavage: after approximately 32 divisions, cells take on a granulated appearance and the outline of the embryo begins to take shape. F) Epiboly: blastodisc expands over the surface of the yolk and the cells formed during cell cleavage begin to form different tissues. G) End of Epiboly: embryo has reached the eyed stage of development. H) Organogenesis: development of fins, circulatory system and external structure, such as operculum.

1.5 Factors affecting egg quality during multiple stages of development

A great deal of evidence exists concerning what are thought to be the major determinants of egg quality in fish (Brooks *et al.*, 1997). Reviews on this subject have identified and categorised factors which may affect egg quality under 4 main themes, these are; environmental factors, husbandry practices, genetic factors and nutritional factors (Bobe and Labbe, 2010; Brooks *et al.*, 1997). Each of these factors are also able to influence egg quality both pre and post ovulation.

1.5.1 Environmental factors affecting egg quality

Comparisons between wild and farmed fish populations have consistently shown that egg quality is higher in wild fish compared to farmed stocks and that the superior quality of wild eggs over farmed eggs is largely due to environmental influences (Brooks *et al.*, 1997; Srivastava and Brown, 1991). Environmental factors which may affect egg quality in fish include the physiochemical conditions of the water (temperature and salinity) and the photoperiod under which the fish are reared.

Temperature is known to have a significant impact on egg quality both before and after ovulation (Brooks *et al.*, 1997). Both high and low temperatures may affect the metabolism of female broodstock during sexual maturation and embryogenesis (Kinne and Kinne, 1961). For example, eggs obtained from female Arctic charr (*Salvelinus alpinus*) held at 5^{o}C had significantly higher survival rates compared to eggs produced by arctic charr held at 10^{o}C (Gillet *et al.*, 1996). In salmonids, the temperature at which eggs are incubated has a significant impact on survival; excessively high or low temperatures can lower egg survival rates during early embryonic stages (Brooks *et al.*, 1997). Previous studies have shown that an abnormally high temperature (12-15^{o}C) during sexual development significantly lowers egg survival rates and increases the probability of embryonic deformities occurring in eggs from Atlantic salmon (Aegerter and Jalabert, 2004; Hokanson *et al.*, 1973; Pankhurst *et al.*, 1996). The mechanisms behind the influence of temperature on egg quality pre and post ovulation are unknown, however Moreau *et al.*, (1991) observed that profound changes in the

pattern of gene expression in cells and tissues can be induced by small fluctuations in temperature and that these changes are likely to affect egg development.

For anadromous fish species, the water salinity experienced during the reproductive season has been shown to have a major impact on reproductive success (Bobe and Labbe, 2010). For example, holding female Atlantic salmon in sea water during the freshwater stage of their reproductive cycle resulted in delayed egg development or egg retention within the ovary (Haffry *et al.*, 1995). Silver perch eggs (*Bidyanus bidyanus*) transferred into increasing concentrations of salt water after fertilisation resulted in decreased egg survival rates. However, at low salinities (6ppt) the eggs showed a significantly higher hatching rate compared to control eggs incubated in freshwater (Guo *et al.*, 1993).

Photoperiod manipulation is a method used to delay or advance egg production in aquaculture (Brook *et al.*, 1997). The effect of artificially manipulating the photoperiod on the reproductive development of broodstock and subsequently egg quality is difficult to assess, as light manipulation can result in the modification of other parameters such as rate of sexual maturation and body size of the fish as well as the water temperature (Bobe and Labbe, 2010). Despite these complications, previous studies have shown that manipulating the photoperiod of farmed salmonids, to either advance or delay spawning, resulted in significantly lower egg survival rates compared to eggs produced by individuals which had experienced a natural photoperiod during their reproductive cycle (Bonnet *et al.*, 2007; Dabrowski and Blom, 1994).

1.5.2 The effect of husbandry practices on egg quality

There is little doubt that sub-optimal husbandry can result in poor reproductive success of farmed fish, however to date few studies have examined the impact this could have on egg quality (Brooks *et al.*, 1997). Key factors which are likely to influence egg quality include; to what extent the fish is stressed, post ovulatory ageing and egg handling post ovulation and fertilisation (Bobe and Labbe, 2010; Brooks *et al.*, 1997). Some authors have also indicated that spawning induction, using hormonal analogues, may also affect egg quality, these studies were not able to clearly identify commercially significant affects

within the family Salmonidae (Arabaci *et al.*, 2004; Breton *et al.*, 1990; Gillet *et al.*, 1996; Mylonas *et al.*, 1992). Indeed, the practice of inducing spawning in salmonids is still considered a standard husbandry practice in the aquaculture industry, but only during the appropriate stage of their life cycle.

Data concerning the effect of stress on reproducing female fish are scarce and different conclusions have been reached depending on the type and intensity of the stressor, the species and at what stage during the reproductive cycle the stressor was applied (Bobe and Labbe, 2010). In spite of these confounding factors some studies have found significant effects of broodstock stress on egg quality. For example, in rainbow trout, repeated bouts of acute stress induced by exposure to air for 3 minutes at regular intervals 9 months prior to spawning resulted in lower egg survival rates (Campbell *et al.*, 1992).

Post ovulatory ageing is caused by the significant morphological and physiological changes which occur to the egg between its release from the ovary, into the coelomic cavity and when it is spawned and fertilised. If an egg is not released from the body cavity and fertilised as soon as possible after ovulation it may lead to a decrease in the eggs ability to be fertilised (Bromage *et al.*, 1994). The rate at which egg quality decreases post ovulation is dependent on the species, environmental factors (mainly temperature) and is subject to high inter-female variation (Bobe and Labbe, 2010). Unlike many other families, cyprinids and salmonids are able to retain the eggs within their body cavity for a number of days without post ovulatory ageing significantly affecting egg quality (Bobe and Labbe, 2010). For example, rainbow trout eggs were assessed for their ability to be successfully fertilised at regular time intervals for up to 4 weeks post ovulation. Aegerter and Jalabert, (2004) stated that during this study maximum egg quality, as defined by the eggs ability to be fertilised, was reached 5 days post ovulation and that after this period egg quality significantly deteriorated. Additional studies have shown that there is a significant decrease in egg survival rates when eggs are stripped and fertilised 7 days after they are ovulated (Bonnet *et al.*, 2007; Bromage *et al.*, 1994).

Within the aquaculture industry eggs are stripped from females after ovulation for the purpose of artificial fertilisation (Bobe and Labbe, 2010). If eggs are not fertilised immediately then the success of future fertilisation depends on the egg

incubation procedures which in turn are highly dependent on several parameters including temperature, oxygenation and the physio-chemical characteristics of the storage media (Bobe and Labbe, 2010; Brooks *et al.*, 1997). For the latter parameter, it is common for eggs to remain within the ovarian fluid in which they were stripped. Eggs stored in the ovarian fluid may remain viable for days without any detrimental impacts on egg quality. In salmonids, unfertilised eggs could be held in vitro in ovarian fluid at 0-2^{o}C for at least 5-7 days without affecting egg quality (Babiak and Dabrowski, 2003; Billard and Gillet, 1981).

1.5.3 The effect of maternal genetics on egg quality

Authors examining the effect of maternal genotypes on fertility in mammals show that genetics can strongly influence egg quality (Ezra *et al.*, 1992; Almeida and Bolton, 1993). Major causes of poor egg quality in mammals are immature or morphologically abnormal eggs, yet some eggs show neither of these characteristics but still fail to develop (Ezra *et al.*, 1992). A number of studies in fish indicate that maternal genetics may have significant impact on egg quality (Brauhn and Kincaid, 1982; Reinitz *et al.*, 1979; Withler, 1987). For example female rainbow trout which produced high quality eggs during their first spawning season did so again during the subsequent season (Brooks *et al.*, 1997). Despite these findings, the effects of maternal genetics on egg quality remains poorly documented (Bobe and Labbe, 2010). The female broodstock selection process within the aquaculture industry is mainly based on the egg survival rates of the female progenitor and therefore loosely based on genetics.

1.5.4 The effect of broodstock nutrition on egg quality

Diet has received the greatest attention with respect to its effects on egg quality (Bobe and Labbe, 2010; Brooks *et al.*, 1997; Kjorsvik *et al.*, 1990; Izquierdo *et al.*, 2001). Dietary components as diverse as lipids, fatty acids, protein, vitamins and carbohydrates have all been shown to affect both the development of the embryo to key stages and egg survival to hatching (Harel *et al.*, 1994; Palace and Werner, 2006; Washburn *et al.*, 1990; Wantanabe *et al.*, 1997).

The majority of documented research concerning the effect of nutrition on egg quality has focused on bulk dietary components such as proteins, lipids and carbohydrates (Izquierdo *et al.*, 2001). Increased concentrations of lipids in the diets of European sea bass (*Dicentrarchus labrax*), increased levels of n-3 fatty acids and improved egg survival rates (Carrillo *et al.*, 1995). Previously, Harel *et al.*, (1994) had found a correlation between levels of n-3 fatty acids in the diet and the concentration of lipid in the eggs produced by gilthead seabream (*Sparus aurata*). The main source of energy in fish is provided by protein and lipid, with dietary carbohydrates being poorly utilised (Walton and Cowey, 1982). Nevertheless, rainbow trout fed a diet deficient in carbohydrate concentrations were found to produce eggs with significantly lower survival rates (Washburn *et al.*, 1990). As well as providing energy to the broodstock, proteins are an important source of amino acids and other essential materials required for embryogenesis (Metcoff 1986). In some species of teleosts, diets rich in protein increased reproductive performance by increasing the availability of molecules such as vitellogenin and altering plasma hormone levels of gonadotrophin releasing hormone (GTrH) and gonadotrophin II (GtH II) which are active during oocyte maturation and ovulation (Navas *et al.*, 1997).

There have been relatively few comprehensive studies on the so-called ‘minor’ dietary constituents (Brooks *et al.*, 1997). The most detailed studies on nutritional trace components have focused on vitamins (Blom and Dabrowski, 1995; Brooks *et al.*, 1997; Dabrowski and Blom, 1994). These studies showed that fish fed a diet deficient in vitamin C produced eggs with significantly lower survival rates compared to fish fed a vitamin C enriched diet. Palace and Werner, (2006) stated that vitamins E and A are essential for the normal development of fish embryos and that as fish are unable to synthesise either of these vitamins their presence in the diet of cultured species is of paramount importance to the issue of egg quality. Recent studies concerning the benefits of trace elements, such as selenium (Se) and zinc (Zn), to the health and reproductive capabilities of domesticated mammals and birds have resulted in similar investigations on the importance of dietary trace element supplementation and its effect on farmed fish (Rider *et al.*, 2009; Rider *et al.*, 2010; Burk 2007; Thorarinsson *et al.*, 1994).

1.6 The effect of dietary selenium supplementation on egg quality

Like humans and other mammals, fish require trace elements for essential biological processes such as, metabolism, growth and reproduction. These minor dietary constituents are responsible for skeletal formation, maintenance of colloidal systems, regulation of acid base equilibrium and for the functionality of biologically important compounds such as hormones and enzymes (Watanabe *et al.*, 1997). In recent years there has been particular interest in the role of the trace element Se in the maintenance and promotion of animal health. Data concerning the biological role of Se and its availability in cultured fish stocks including broodstock is fragmentary and limited, while information on the maternal transfer of dietary Se to eggs and the subsequent effect on egg quality remains undocumented.

1.6.1 Chemistry and biochemistry of Se in fish

Se, atomic number 34 and atomic weight 78.9, belongs to the elemental group of non-metals which also includes carbon (C), nitrogen (N), phosphorous (P) and sulphur (S). In nature, Se is found in its inorganic form as selenite, selenate and selenide (Daniels 1996). At present Se supplementation is not common place in cultured fish diets, and any increase in Se concentrations is by request and largely in the form of the inorganic Se salt, sodium selenite (Na_2SeO^3) (Hilton *et al.*, 1980). In animal and plant based feedstuffs Se is predominately organically bound by the substitution of sulphur by Se in the amino-acids cysteine and methionine to form selenocysteine (Se-Cys) and selenomethionine (Se-Met) respectively (Daniels 1996). In addition to individual amino-acids, organic Se is also found in Se-yeasts which predominately contain Se-Met but also Se-Cys, Se-methyl-selenocysteine, γ-glutamyl-Se-methyl-selenocysteine and Se-adenosyl-selenohomocysteine (B'Hymer and Caruso, 2000; Schrauzer 2003).

The dietary requirement of Se, in the form of sodium selenite, for rainbow trout has been estimated to be 0.35mg/kg based on the activity of the Se containing enzyme, glutathione peroxidase (GSH-Px) within the plasma (Arthur 2000; Hilton *et al.*, 1980).The bioavailability of the different forms of Se however varies significantly (Rider *et al.*, 2009). This is of great importance in the aquaculture

industry as according to the EU additive directive (Regulation (EC) 1831/2003), Se supplementation levels are limited to a maximum of 0.5mg/kg, however Se ingested and deposited in the tissue is not necessarily available for metabolic processes. Organic forms of Se, such as selenomethionine and selenocysteine are synthesised by various plants, including yeasts and marine algae, and is generally far more biochemically available than inorganic forms (Bell and Cowey, 1989). For example, Rider *et al.*, (2009) found that rainbow trout fed a Sel-plex supplemented diet (a standard industry diet enhanced with Se enriched yeast) contained higher concentrations of Se in all tissues recorded, except the liver and pyloric caeca. Se bioavailability, measured using Se-dependant antioxidant activity, was also increased in comparison to the fish which had received dietary sodium selenite supplementation.

Knowledge concerning Se metabolism in fish is fragmentary and dependent on a number of variables, including, route of entry (dietary v waterborne), type (inorganic v organic) and the concentration of Se present (Hilton *et al.*, 1982; Hodson *et al.*, 1980; Hodson and Hilton, 1983). Each of these factors should be considered prior to supplementation taking place. For example, fish can absorb inorganic Se via the gills. Upon absorption via this route the Se is transported in the plasma to all organs except the liver. This is in contrast to Se absorbed by ingestion which is transported to the liver by the circulatory portal (Hodson and Hilton, 1983). Like humans and other mammals, the highest store of Se in fish occurs in the kidney, however at dietary levels above 0.35mg/kg the liver contains the highest Se levels (Hilton *et al.*, 1980; Hilton *et al.*, 1982).

1.6.2 The effects of Se on fish health

The majority of selenoproteins identified so far have significant roles in cellular antioxidant activity. Enzymes containing Se, such as GSH-Px and thioredoxin reductase (Txr-R) catalyse the reduction of reactive oxygen species (ROS) (Arthur 2000; Arteel and Sies, 2001; Halliwell and Gutteridge, 1999). Consequently Se is considered important in controlling endogenous ROS produced during normal aerobic metabolism, calorific deficiencies, increased physical activity, stress and exogenous sources such as ionising radiation. For example, Rider *et al.*, (2009) found that rainbow trout fed the Se yeast, Sel-plex®, under

artificially stressed conditions had lower hepatic lipid peroxidation levels compared to fish fed a standard industrial diet.

Research concerning the effect of Se on fish heath has been limited and the actual benefits confounded by its synergistic effects with vitamin E, another well-known antioxidant. Juvenile chinook salmon (*Oncorhynchus tshawytscha*) infected with *Renibacterium salmoninarum* and fed a diet containing high levels of vitamin E and sodium selenite had a significantly higher survival rate compared to infected fish given a low vitamin E and sodium selenite diet (Thorarinsson *et al.*, 1994). However, salmon receiving either a high vitamin E + low Se or low vitamin E + high Se diet showed no significant difference in survival rates. It therefore remains to be established whether the significantly higher survival rates were due to the vitamin E or the sodium selenite.

It is well known that oxidative stress, caused by an increase in the levels of ROS within the cells is detrimental to fish health due to the loss of cell function eventually leading to apoptosis (Pascual *et al.*, 2003; Rodriguez *et al.*, 1993). It has been strongly indicated that oxidative stress, induced by sub-lethal exposure to exotic chemicals in the water, can cause growth deficiencies in some species of fish. For example, chronic, sub-lethal concentrations of fullerene aggregates caused oxidative stress in goldfish (*Carassius auratus*), determined by the induction of enzymatic and non-enzymatic antioxidants, and a significant decrease in both body weight and length compared to these held in normal fresh water (Zhu *et al.*, 2008). Rider *et al.*, (2009) also identified that Se utilisation in rainbow trout held under farmed conditions and subjected to standard aquaculture stressors increased, and that this was related to elevated GSH-Px activity within the liver. This study concluded that dietary Se supplementation was essential for the health of fast growing juvenile salmonids held under farmed conditions.

Trace element requirements of fish, like all farmed animals, must be accurately determined so that dietary supplementation maintains homeostasis and promotes continued biological functionality. The possibility of excess Se supplementation is of great concern due to the narrow range at which dietary Se concentrations turn from being nutritional to toxic. Hilton *et al.*, (1982) found that in salmonids, dietary Se becomes toxic at approximately 10 times that of

normal dietary levels (1-2mg/kg) and that symptoms of Se toxicity included reduced growth rates, poor feeding efficiency and high number of mortalities. However, there may be differences between organic and inorganic Se toxicity in supplemented fish feeds. For example, chinook salmon, given an organic Se supplementation 18 times that of normal dietary levels, showed no signs of toxicity (Hamilton *et al.*, 1990)

1.6.3 The maternal transfer of Se and the effect on egg quality

Information regarding the presence of Se in animal gametes has focused on the concentration of this trace element in the eggs of mammals and birds. These studies found that there is a significant amount of variation regarding the location of Se within eggs, particularly bird eggs. For example, Se was found to be present in all parts of the chicken egg (*Gallus gallus*), the highest concentration was found in the shell membrane while the Se content of the shell itself was comparable to that of albumin (Surai *et al.*, 2004). Alternatively the concentration of Se in the quail shell represents a significant amount of the total egg Se content, while the shells of roseate terns (*Sterna dougalli*) and herring gulls (*Larus argentatus*) provided a minimal amount of the total egg Se content (Burger 1994; Surai *et al.*, 2004).

The majority of information regarding the effect of dietary Se on egg quality has been undertaken in birds as well. Authors have also found that dietary Se is maternally transferred into the egg yolk of several species, including rats, chickens and ducks (Scott *et al.*, 1977; Thompson and Scott, 1970; Dean and Combs, 1981). Egg freshness, measured in Haugh units, is one of the most important parameters determining consumer perception and demand within the poultry industry (Surai 2006). During storage, poultry egg freshness decreases. Previous studies have indicated that the inclusion of Se into the diet of layer hens significantly moderated the decline in egg freshness after 2 weeks of storage compared to eggs from hens fed a control diet (Pappas *et al.*, 2005; Burk 2006). Furthermore, it has been suggested that organic Se supplemented into the breeder diet of hens also improved egg survival, demonstrating possible embryonic benefits (Edens and Sefton, 2003; Sefton and Edens, 2004). Previous studies using poultry have indicated that the benefits of dietary Se supplementation extent beyond the embryonic stage of development. For

example Surai (2000), found that an increase in Se concentrations in the diet of Cobb broiler breeder hens significantly increased the levels of Se found within the yolk, and that this may have contributed to the significant increase in Se-dependent GSH-Px concentrations in the liver of 1-day old and 5-day old chicks. This study found that the increase in the GSH-Px antioxidant activity significantly decreased the chick livers susceptibility to peroxidation.

Furthermore, the eggs from layers fed organic Se supplemented diets have also been reported to exhibit better shell quality characteristics than those which were provided with a non-supplemented diet (Wakebe 1999; Pavlovic *et al.*, 2009). For example Paton *et al.*, (2000) found that laying hens fed an organic Se supplemented diets produced eggs with increased shell breaking strengths compared to cohorts fed a standard industry diet. The potential benefits of dietary Se supplementation and its effects on chicken egg quality are therefore well established; however whether these effects can also be transferred onto fish and their eggs remains to be seen.

1.7 Egg adhesion in fish

Many teleost fish species produce adhesive eggs, which attach to plants or hard substrates, such as rock and gravel (Mansour *et al.*, 2009b). This mechanism prevents the eggs from being swept away by water currents, ensuring they remain within the optimal incubation environment selected by the parents (Mansour *et al.*, 2009a; Mansour *et al.*, 2009b). Furthermore, studies suggest that species of fish which produce clumps or strings of adhesive eggs, decrease the probability of their eggs being attacked by aquatic predators (Mansour *et al.*, 2009b; Reihl and Patzner, 1998). Egg adhesion mechanisms can be broadly classified into three categories, (1) mechanical structures on the outer layer of the chorion, the zona radiata externa (ZRE), (2) specific biochemical compounds on the surface of the chorion, or (3) eggs can have both mechanical structures and specific biochemical compounds (Reihl and Patzner, 1998).

Mechanical egg adhesive mechanisms have been indentified in a number of teleost species. For example, attachment filaments are found on eggs produced by species within the families cichlidae, gobiidae and blenniidae (Reihl and Patzner, 1998). Attachment filaments are long string-like structures located on

the chorion, at the animal or vegetal pole of the egg. This mode of attachment allows them to be openly deposited and remain on a wide variety of substrates (Meijide and Guerrero, 2000). Alternatively, Mansour *et al.*, (2009b) found that glycoproteins, originating from the ZRE, formed an adhesive layer around the eggs produced by common carp (Cyprinus carpio) and that this biochemical mechanism enables eggs to remain within optimal spawning areas. The eggs of dace (*Leuciscus leuciscus*) contain both mechanical and biochemical attachment mechanisms. Short, villi-like structures were identified on the surface of the chorion, while histochemical analysis of the chorion itself produced a positive result for the presence of glycoproteins (Reihl and Patzner, 1998).

Information regarding the presence of adhesive mechanisms on fish eggs is often species specific and the variety of mechanical and/or biochemical alterations to the chorion of adhesive eggs between species makes comparisons difficult (Reihl and Patzner, 1998).Furthermore, details regarding the reproductive physiology, which leads to egg adhesion and the underlying mechanisms which produce the biochemical activity remain poorly documented, therefore, additional studies on these subjects are needed to understand the processes leading to egg adhesion.

1.8 Aim of current study

The main aim of this study is to evaluate and develop a range of novel methods of assessing fish egg quality in salmonids and determine how changes in nutritional and environmental factors experienced by broodfish affect these measurements. In addition, this study will also investigate adaptations in the eggs of critically endangered salmonids and how these changes to the fundamental biological and morphological characteristics of the egg may aid survival in high energy environments. Specifically the work described in this thesis aims to deliver the following information:

1. To compare various novel and previously examined parameters of egg quality, with the aim of selecting methods which are useful to determining egg quality.

2. Determine the effect of broodstock holding environment on brown trout egg quality.

3. To examine egg quality in Atlantic salmon held under commercial conditions.

4. Examining the maternal transfer of dietary Se to eggs in Atlantic salmon held under commercial conditions, and investigate what effect dietary supplementation has on egg quality in terms of egg survival and the protein structure of the chorion.

5. To determine the role of Se supplementation of broodstock diet on *Saprolegnia* resistance in eggs.

6. Undertake preliminary investigations into the structural and biochemical contents of the egg chorion in European whitefish (*Coregonus lavaretus*) from Loch Eck, focusing on possible adhesive adaptations found within this population.

2 Analytical procedures for defining fish egg quality

2.1 Abstract

After egg ovulation, brown trout broodstock held at two different locations were stripped and the eggs used to define and determine parameters of egg quality. Based on its use within the aquaculture industry, egg survival rates were deemed an accurate ultimate measure defining egg quality. Linear regression showed a positive relationship between egg survival and chorion breaking strength, the volume of eggs produced by individual broodstock and elemental concentrations within the egg. A literature review discounted the biological significance of using fecundity as a parameter of egg quality but literature did provide evidence of the advantages of using proteomic analysis. This chapter successfully provided an industrial and biologically useful definition of egg quality while providing relevant parameters for determining reproductive success which will be further investigated in the thesis.

2.2 Introduction

The ability to manipulate reproduction is an essential requirement in aquaculture and is regularly utilised in order to produce a yearly supply of eggs; however despite this ability, one of the major factors limiting the expansion of the industry is the significant amount of variation observed in egg quality (Bromage *et al.*, 1992; Lahnsteiner *et al.*, 1999). In the cultivation of fish for commercial purposes, efforts to improve egg quality have often been carried out on a variety of freshwater and marine species including Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), arctic charr (*Salvelinus alpinus*), Atlantic cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*) and gilt-head seabream (*Sparus aurata*) (Brooks *et al.*, 1997). Yet there are a number of issues which have hindered efforts to improve egg quality (Bobe and Labbe, 2010). One of the main problems is that the definition of egg quality is not always consistent among existing studies. This has resulted in several types of indicators being used, which makes comparisons between studies difficult. In addition, previous investigations have attempted to use a variety of parameters to estimate egg quality and despite some studies showing significant

relationships between constituents of the egg and measures of reproductive success, egg quality in wild or captive held fish is influenced by many factors and the relative effects of each of these factors on egg parameters and egg quality is highly variable (Bobe and Labbe, 2010).

Embryonic survival at specific developmental phases is one of the most common ways of characterising egg quality (Bobe and Labbe, 2010). Survival can thus be assessed at specific stages, such as the eyed stage and hatching, which can be easily identified in fish. The aquaculture industry defines egg quality as the ability of an egg to be successfully fertilised, reach key embryonic stages of development and subsequently hatch, but in terms of commercial importance, egg survival rates i.e. the number of eggs reaching the hatching stage, is perhaps the most important definition (Bromage *et al.*, 1992; Brooks *et al.*, 1997). The majority of studies investigating reproductive success in teleost species have used egg survival rates to define egg quality; however others have selected earlier developmental stages for their definition. The situation is further complicated by the same authors using different definitions of egg quality during alternative studies. For example, during earlier studies Lahnsteiner *et al.*, (1999; 2000; 2001; 2005) used egg viability, or in other words, the fertilisation rate of eggs produced by fish species, to characterise egg quality. However, the same author using similar species of fish altered their definition of egg quality to the egg's ability to reach the eyed stage of development, during a later investigation (Lahnsteiner *et al.*, 2007).

Previous studies have examined a variety of egg parameters which may be used to determine egg quality in cultured fish during early stages of their development. Parameters as diverse as egg weight, chorion weight, egg iron levels, lipid composition and protein concentrations have all been used to determine egg quality in teleost species (Bobe and Labbe, 2010; Brooks *et al.*, 1997; Kjorsvik *et al.*, 1990; Lahnsteiner *et al.*, 1999). Despite a number of these parameters being successfully related to egg quality, there is a lack of consistency in results between studies examining the same parameters. For example, Mansour *et al.*, (2007) found that lipid droplet distribution in brown trout (*Salmo trutta*) eggs was significantly related to egg quality, defined as the number of eggs reaching the eyed stage of development. During an alternative study in rainbow trout, Ciereszko *et al.*, (2009) found that there was no

consistency in the relationship between lipid droplet distributions and egg quality. A number of factors, such as diet, temperature, husbandry, stress, genetics, and species may influence egg quality and cause inter-study variation in the results between investigations (Brooks *et al.*, 1997; Bobe and Labbe, 2010).

Information gathered from literature associated with reproductive success in wild and farmed fish, suggest that studies need to first select and retain their definition of egg quality throughout an entire investigation. Next biological and industrial relevant parameters of egg quality must be chosen which can accurately determine reproductive success during the early phases of embryonic development. For example, the chorion from fish is a proteinaceous membrane which protects the egg from mechanical damage during spawning and embryonic development (Arukwe and Goksoyr, 2003), changes in the protein content of the chorion may adversely affect egg survival rates, and hence studies which examine this parameter would be considered as exceptionally pertinent for determining egg quality. With this in mind, a number of comparative procedures were carried out to assess how best to define and compare determinants of egg quality as a prelude to carrying out the main studies reported in this thesis.

2.3 Materials and methods

2.3.1 Source of eggs

The eggs used in these preliminary investigations were sourced from brown trout held at Ae Fishery (Location 1) and S.C.E.N.E. (Location 2) and for the purpose of the first study carried out as part of this thesis, details of which can be found in Appendix A sections A.2.1, A.2.2 and A.2.3.

2.3.2 Egg survival

Approximately 500 eggs (estimated by weight) were collected from 7 females held at Location 1 and 10 females held at Location 2. These were then sub-divided into two replicates and fertilised by a single male. Eggs were then activated and placed into individual custom built incubation trays. Egg activation, in this context, occurs when eggs are fertilised and come into contact with water. The difference in osmolarity between the inside of the egg

and the water causes an influx of water into the perivitelline space of the egg. This causes the eggs to swell in size and a cross-linking of chorion proteins (Oppen-Berntsen *et al.*, 1990; Rudy and Potts, 1969). Incubation trays were constructed from plastic mesh (5mm mesh diameter) wrapped round a solid square Perspex base and rim (10cmx10cmx15cm). These trays were placed in a 200L flow-through tank (5L min^{-1}) containing two water filters (Fluval A460). The eggs in individual trays were checked every alternate day for mortalities. Dead eggs were identified by their white/opaque appearance, recorded and removed from the incubation system. The experiment was terminated when all eggs reached the eyed stage of development. Water temperature during egg incubation ranged between 5°C and 14°C (mean= 8°C). Egg survival was calculated as the mean number of eggs expressed as a % of 500 remaining within both replicate incubation trays after eggs had reached the eyed stage of development.

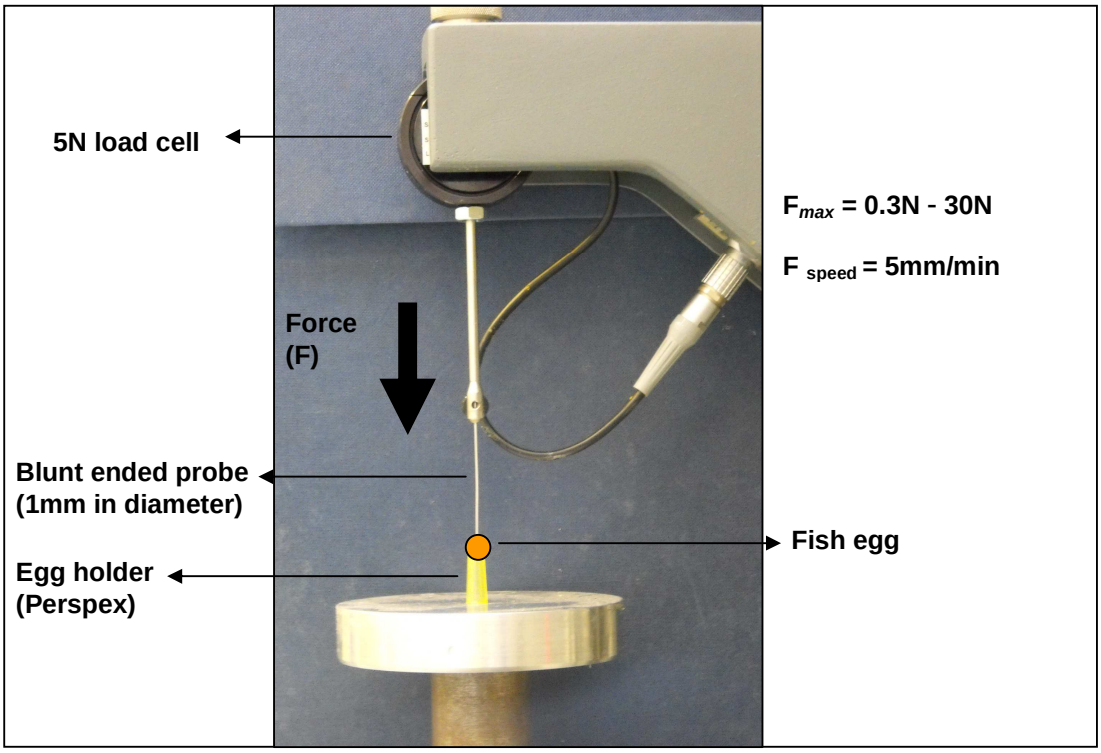

Figure 2-1: The Lloyd materials testing device and set up used to test the chorion breaking strength.

2.3.3 Chorion breaking strength

The chorion breaking strength was measured using a Lloyd LRX compression test instrument (Ametek Inc) using the test set up shown in (Figure 2-1). A 1mm

diameter blunt ended probe connected to a 5N load cell was applied to the surface of the egg and lowered at a speed of 5mm/min until the egg ruptured. The applied force was recorded throughout the test. The breaking strength was recorded as the maximum load recorded before rupture (Figure 2-2).

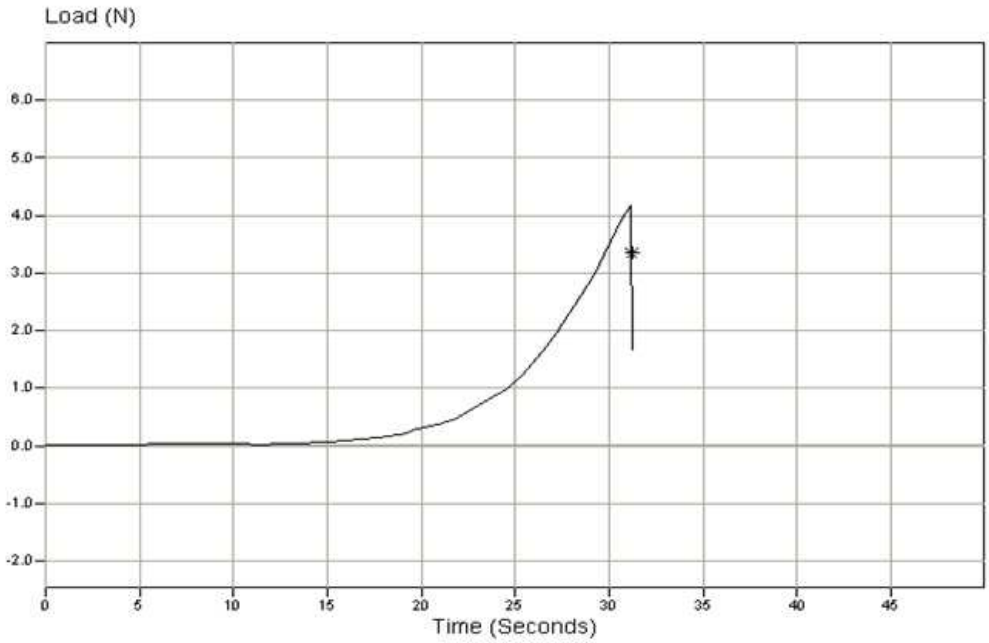

Figure 2-2: Typical graphical display of the chorion breaking strength from a brown trout egg tested using the Lloyds compression test instrument. When the chorion ruptures there is a sharp drop in resistance (indicated at *). The maximum force applied (N) was defined as the breaking strength of the chorion.

2.3.4 Egg chorion and yolk elemental concentrations

Egg chorions for elemental analysis were prepared as follows: Eggs were gently punctured with a sterilised needle. The yolk was extruded by gentle manipulation of the chorion. Each chorion was then placed in individually marked well-plates, wrapped in tinfoil and dried at 37^{o}C for 24 hours.

For elemental analysis of the egg yolk, additional whole eggs were placed in individually marked well-plates, wrapped in tinfoil and dried at 37^{o}C for 24 hours. After drying, the eggs were halved so that the yolk-filled centre of the egg was exposed.

Both the chorion and yolk samples were then mounted onto aluminium stubs using double sided sticky carbon tabs (Gisbourne Microscopy Services). Yolk samples were placed onto the tabs so that the exposed yolk was facing upwards.

Analysis of elemental concentrations was then carried out by energy-dispersive x-ray spectroscopy (E.D.X.), using a Philips XL30 ESEM equipped with a Phoenix energy dispersive x-ray detector (operating voltage = 20kv, working distance = 10mm) at the General Microscope Facility (University of Dundee). A typical spectrum is shown in Figure 2-3. The percentage concentration of each of the major elements detected was derived using the fundamental principle that each element has a unique atomic signature allowing X-rays that are characteristic of an elements atomic structure to be uniquely identified from one another (Allen 1973). The intensity of the energy released by each element when excited by the X-ray beam appears as a peak. The size of the peak for each individual element is proportional to the concentration present; allowing what percentage of a specific element constitutes the whole sample to be extrapolated.

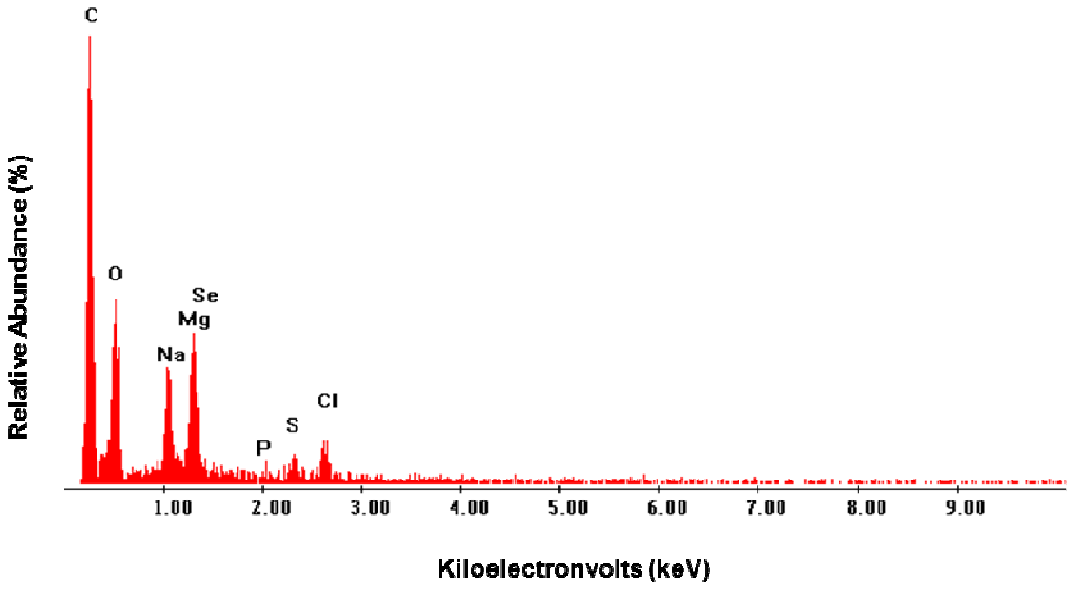

Figure 2-3: Example of the elemental frequency data which is subsequently transformed into percentages by the Phoenix energy dispersive x-ray detector.

2.3.5 Fish Fecundity

The fecundity of individual female broodstock was estimated after the females had been stripped, as follows: ovarian fluid was drained from the eggs using a metal sieve (1mm mesh diameter). All eggs stripped from an individual were weighed to measure the mass of eggs produced. The number of eggs was estimated using the weight of 10 eggs calculated against the weight of the entire

egg mass. The estimated egg numbers and the volume of eggs were used as a measure of fecundity.

2.3.6 Egg weight and diameter pre and post water hardening

Ten eggs from each individual female were removed from the egg mass in order to measure their weight and diameter before and after water hardening. Each egg was weighed to the nearest 0.1mg and its diameter (mm) measured with digital callipers to the nearest 0.1mm. Eggs were then water hardened in 100ml of water (between 4-6^{o}C) for exactly 3hr. Egg water hardening, in this context, occurs when unfertilised eggs come into contact with water. The difference in osmolarity between the inside of the egg and the water causes an influx of water into the perivitelline space of the egg. This causes the eggs to swell in size and a cross-linking of chorion proteins (Oppen-Berntsen *et al.*, 1990; Rudy and Potts, 1969). Their weight and diameter was subsequently re-measured.

2.3.7 Chorion protein analysis using SDS gel electrophoresis

The protein composition of the chorion was investigated using sodium dodecyl sulphate (SDS) gel electrophoresis. Chorions from ten non-water hardened eggs from each female were prepared as follows. First the eggs were punctured with sterilised forceps, and the yolk removed via manipulation of the chorion. The ten chorions from each female were then pooled and macerated in 1ml of homogenisation buffer (1M Tris pH 7.4; 10% SDS; ultra-pure water; protease inhibitor cocktail (Sigma Life Sciences)). The pooled samples were subsequently transferred to a 1.5ml eppendorf and stored at -80^{o}C until required.

The protein concentration of each pooled chorion sample was measured using a modified version of the Lowry assay (Lowry *et al.*, 1951). Samples were prepared using 1ml of BCA reagents (Thermo Scientific), which enables the determination of results in a protein dependant colorimetric reaction. Samples were incubated at 37^{o}C for 30 mins in a water bath then transferred to cuvettes and light absorbance read at 562nm using a CE-1011 spectrophotometer (Cecil Instruments Ltd). A bovine serum albumin (BSA) kit (Thermo Scientific) was used as a standard to estimate the concentration of protein in each chorion sample.

In order to carry out SDS gel electrophoresis, 1µg/ml (dilute sample) and 10µg/ml (concentrated sample) of protein was transferred from the pooled sample to a 1.5ml eppendorf with 8µm of three times sample buffer solution (3x sample buffer and 1M dithiotreitol (DTT)) and ultra-pure water to make a final volume of 25µl. Samples were then heated at 90^{o}C for 4 mins (Tempette Junior TE-8J water bath). Pre-cast NuPage 4-12% bis-tris, 10 well, 1.0mm thick gels (Invitrogen) were loaded into a Novex mini-cell (Invitrogen) with 20% NuPage MES SDS running buffer (Invitrogen). Approximately 25µl of the dilute and concentrated samples from each female were then pipetted into individual wells along with SeeBlue pre-stained standard (Invitrogen). The SDS gel electrophoresis mini-cell was run at 90v for 2 hours, rinsed in ultra-pure water for 1 minute and placed on a Vibrax-VXR agitator (IKA Ltd) and immersed in 100ml of fixative solution (40% ethanol, 10% acetic acid and 50% ultrapure water) overnight. Gels were subsequently stained with Simply Blue Safe Stain (Invitrogen) and scanned and saved onto the computer.

2.3.8 Lipid droplet assessment

Ten unfertilised, non-activated eggs from each female were photographed at the animal and vegetal pole using a stereo microscope at 15x magnification. Using three independent assessors, the distribution of lipid droplets in each egg was classified into 1 of 4 categories as defined by Mansour *et al.*, (2007) and illustrated in Figure 2-4.

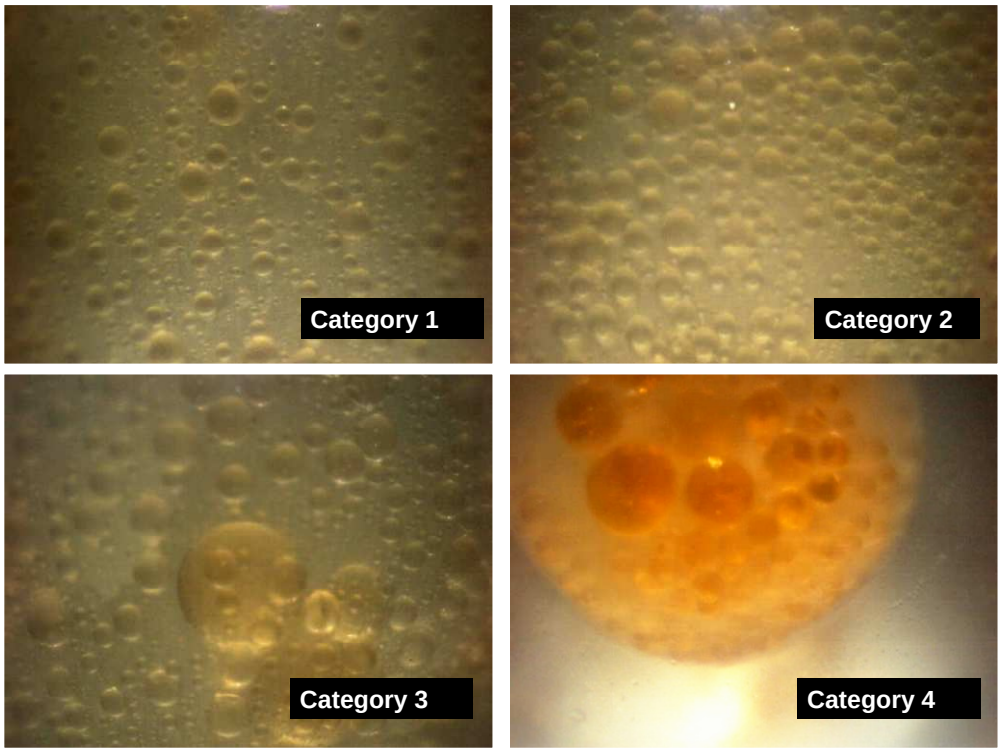

Figure 2-4: Lipid droplet distributions from brown trout eggs. Category 1 (High quality): lipid droplets are evenly distributed throughout the whole egg. Category 2 (High quality): some lipid droplets are coalesced in one pole of the egg. Category 3 (Low quality): most of the lipid droplets are coalesced in two poles of an egg. Category 4 (Low quality): all lipid droplets are coalesced to a big droplet in one pole of an egg.

2.3.9 Data analysis

The relationship between egg quality parameters and egg survival was tested using linear regression analysis, except for lipid droplet distributions which were analysed using spearman rank order correlation. All data presented as percentages were arcsine transformed prior to analysis. The Minitab® 16 was used to analyse the data.

2.4 Results

There is variation in the means of egg quality parameters from fish held at both locations (Table 2-1).

Table 2-1: Descriptive statistics of egg quality parameters from a single group of fish held at two different locations.

Parameters of egg quality	Location 1 (Mn ± Std dev)	Location 2 (Mn ± Std dev)
Non- water hardened breaking strength (N)	0.68 ± 0.63	6.75 ±1.00
Water hardened breaking strength (N)	1.38 ±1.34	20.22 ± 4.27
Pre-activation diameter (mm)	4.49 ± 0.20	4.63 ± 0.31
Post activation diameter (mm)	5.00 ± 0.20	5.15 ± 0.15
Pre-activation weight (g)	0.06 ± 0.01	0.06 ± 0.01
Post activation weight (g)	0.07 ± 0.01	0.08 ± 0.03
Egg weight (g)	192.10 ± 50.30	222.10 ± 86.70
N° of eggs (est)	2902.00 ± 652.00	3329.00 ± 1275.00
Se yolk (%)	3.48 ± 2.47	4.71 ± 0.91
C yolk (%)	50.93 ± 2.88	51.35 ± 2.72
O yolk (%)	33.52 ± 3.35	33.95 ± 3.30
Na yolk (%)	4.50 ± 0.80	4.50 ± 0.30
Mg yolk (%)	4.46 ± 0.64	4.03 ± 0.45
P yolk (%)	7.38 ± 1.75	6.05 ± 1.37
S yolk (%)	7.84 ± 1.81	6.15 ± 1.63
Cl yolk (%)	5.61 ± 1.83	4.82 ± 2.00
K yolk (%)	6.81 ± 2.40	5.31 ± 1.81
Ca yolk (%)	4.47 ± 0.95	3.64 ± 1.36
Se chorion (%)	2.60 ± 1.85	4.10 ± 0.87
C chorion (%)	48.77 ± 1.80	47.69 ± 2.67
O chorion (%)	38.43 ± 2.52	39.55 ± 2.14
Na chorion (%)	4.34 ± 0.70	4.50 ± 0.75
Mg chorion (%)	5.62 ± 4.59	3.73 ± 0.68
P chorion (%)	5.12 ± 1.68	3.67 ± 0.64
S chorion (%)	4.92 ± 1.76	4.12 ± 1.61
Cl chorion (%)	3.39 ± 1.13	3.57 ± 4.22
K chorion (%)	4.15 ± 1.38	2.19 ± 1.01
Ca chorion (%)	3.70 ± 1.50	3.00 ± 3.16
Egg survival (%)	17.42 ± 19.55	58.57 ± 10.55
Lipid droplet distribution (ranked)	1.98 ± 0.84	2.73 ± 0.90

2.4.1 Relationship between individual egg quality parameters and egg survival

For fish held in Location 1 there was a positive relationship between the breaking strength of non-water hardened chorions and egg survival ($F_{[1,6]}$=8.57, r^2= 0.63, p=0.033), however there was also a strong negative relationship between the weight of the eggs produced and egg survival ($F_{[1,6]}$= 9.90, r^2= 0.66, p= 0.025) and sodium yolk concentrations and egg survival ($F_{[1,6]}$= 8.67, r^2= 0.63, p= 0.032) (Table 2-2).

There were no significant relationships between egg quality parameters and egg survival for fish held at Location 2.

Table 2-2: Relationship between parameters of egg quality and egg survival from a single group of fish held at two different locations

Parameters of egg quality v Egg survival	Location 1 p-value	Location 2 p-value
Non-water hardening breaking strength (N)	**0.033**	0.650
Water hardened breaking strength (N)	0.083	0.074
Pre-activation diameter (mm)	0.920	0.763
Post activation diameter (mm)	0.309	0.536
Pre-activation weight (g)	0.190	0.234
Post activation weight (g)	0.127	0.761
Volume of eggs produced (g)	**0.025**	0.297
N° of eggs produced (est)	0.711	0.155
Se yolk (%)	0.288	0.359
C yolk (%)	0.354	0.157
O yolk (%)	0.736	0.396
Na yolk (%)	**0.032**	0.227
Mg yolk (%)	0.109	0.553
P yolk (%)	0.395	0.766
S yolk (%)	0.518	0.861
Cl yolk (%)	0.157	0.262
K yolk (%)	0.302	0.833
Ca yolk (%)	0.266	0.743
Se chorion (%)	0.400	0.849
C chorion (%)	0.762	0.854
O chorion (%)	0.763	0.347
Na chorion (%)	0.284	0.514
Mg chorion (%)	0.373	0.238
P chorion (%)	0.996	0.684
S chorion (%)	0.793	0.170
Cl chorion (%)	0.455	0.263
K chorion (%)	0.895	0.180
Ca chorion (%)	0.274	0.226
Lipid droplet distribution (ranked)	0.606	0.071

2.4.2 Chorion protein analysis using SDS gel electrophoresis

There were no consistent differences in the number of protein bands observed in the chorions from eggs produced by brown trout held at Location 1 or Location 2, during 1D-gel electrophoresis (Figure 2-5).

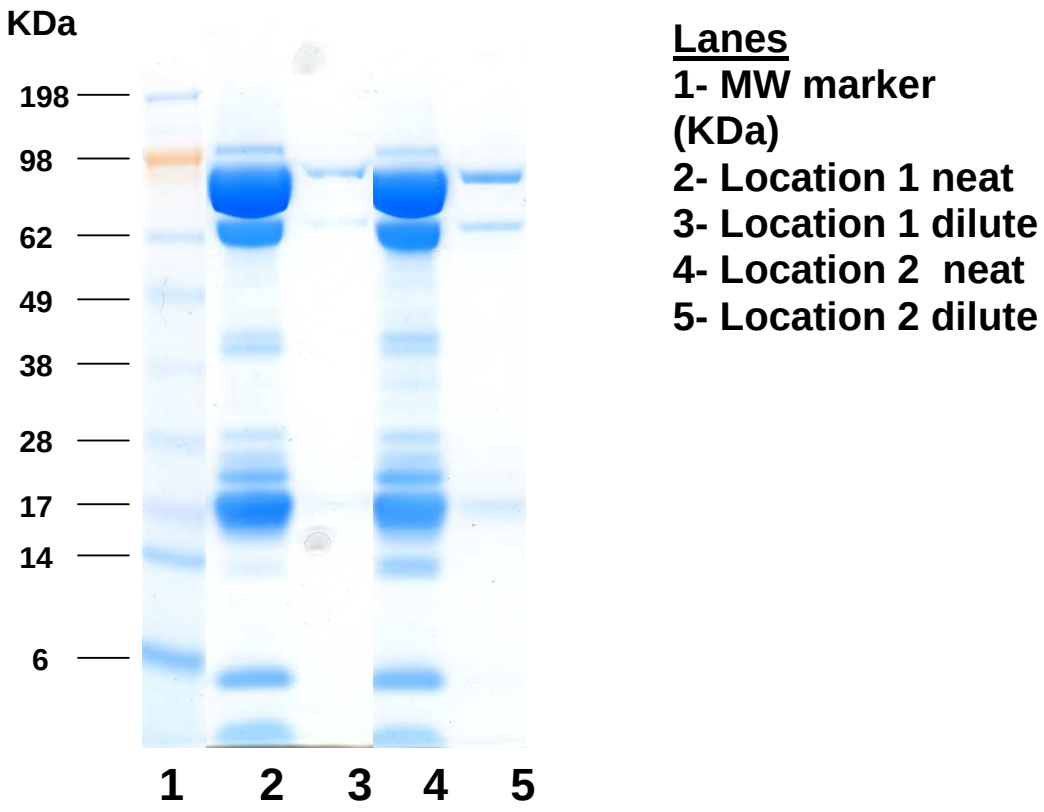

Figure 2-5: Protein profile of the chorion (concentrated and dilute samples) from eggs produced by brown trout held at Location 1 and Location 2.

2.5 Discussion

This preliminary study showed that egg survival, chorion breaking strength, egg elemental concentrations and chorion protein profile provide the most useful information in terms of assessing fish egg quality.

Egg survival was relatively easy to determine in the experimental set up described. However as a measure of egg quality it lacks specific detail concerning what properties of the egg make it 'good' or 'bad'. The aquaculture industry however consistently makes use of egg survival data to assess their

broodstocks reproductive success by comparing egg mortality rates from year to year (Bromage *et al.*, 1992). The definition of egg quality can ultimately be described as the egg's ability to survive or in other words, its ability to be fertilised, successfully reach key developmental stages and subsequently hatch at the end of embryogenesis (Bobe and Labbe, 2010). For these reasons egg survival is used as a measure of egg quality throughout this thesis.

Researchers have previously investigated the mechanical properties of the chorion in various teleost species (Davenport *et al.*, 1986; Iuchi *et al.*, 1996; Zotin 1958). The chorion functions as a mechanical barrier, preventing the embryo within being damaged during development. Without a strong chorion, eggs are likely to become damaged during the processing procedures performed within the aquaculture industry and would result in poor egg survival rates. The results of the chorion breaking strength for brown trout eggs are similar to those reported by Davenport *et al.*, (1986), Iuchi *et al.*, (1996) and Zoltin (1958) for other species of teleosts, therefore, providing confidence in the accuracy of the measurements. In addition, there was a significant relationship between chorion breaking strength and egg survival. For these reasons the chorion breaking strength has been used throughout this thesis when examining egg quality in cultured species.

SDS-gel electrophoresis offers the potential to identify individual proteins in the chorion of fish eggs, which could potentially be used to provide useful biomarkers for egg quality (Arukwe and Goksoyr 2003). The results presented in Figure 2-4 show that a number of discrete protein bands can be visualised. With further improvement in the preparative stages, the semi quantitative and quantitative assessment of the major protein bands present should be possible. For these reasons this method is investigated further in this thesis.

The analysis of individual elemental concentrations in the yolk and chorion by EDX has not previously been used to assess egg quality in salmonids. Many trace elements, such as copper (Cu), zinc (Zn), iron (Fe), manganese (Mn) and selenium (Se) are essential for the health of all animals including fish (Wantanabe *et al.*, 1997). In this preliminary study EDX was able to extrapolate the percentage concentration of multiple elements present within a single fish egg (chorion and yolk). Moreover, it is able to identify subtle changes in the

elemental composition of eggs from individual broodstock held under different experimental conditions (as detailed in Appendix 1, section 1.3.1). For these reasons this methodology has been applied elsewhere in this thesis.

Historically, within the aquaculture industry there has been the perception that fecundity is an important parameter of egg quality, for example, the more eggs produced by females the larger the potential profit for the company (Brooks *et al.*, 1997), however the spawning strategies of some species *i.e.* the Atlantic cod (*Gadus morhua*), suggest that the size of the egg takes priority over fecundity (Kjesbu *et al.*, 1996). Despite the significant relationship between the mass of eggs produced and egg survival, there is relatively little data that can support the hypotheses that the number or size of eggs produced by broodstock in any way corresponds to egg quality (Bobe and Labbe, 2010). For these reasons it was decided not to include this measurement elsewhere in this thesis.

Mansour *et al.*, (2007) observed that the distribution of lipids within the yolk of salmonid eggs was reflective of egg survival, however, the use of such an estimator is limited and the lack of a consistent relationship between lipid distributions and egg quality was recently stressed (Bobe and Labbe, 2010; Ciereszko *et al.*, 2009). The subjective, qualitative analysis of the data leads to inconsistent results (see Appendix 1, section 1.3.3) and therefore this method was not included elsewhere in this thesis.

In conclusion, this preliminary investigation has established that egg survival, chorion breaking strength and egg elemental analysis provide a more holistic approach to assessing fish egg quality and enables the best opportunity of detecting differences in egg quality between individuals and groups of fish receiving different treatments. With further modifications to the methodology, chorion protein analysis may also provide a novel approach to assessing egg quality.

3 Effect of holding environment on egg quality in brown trout (*Salmo trutta*)

3.1 Abstract

Brown trout (*Salmo trutta*) broodstock from a single population were separated prior to spawning and exposed to two different holding environments, a 'S.C.E.N.E. system' and a 'Ae system' at 2 sites. Eggs were stripped from females and 13 measures of egg quality collected, analysed individually, combined by principle components analysis into an integrated egg quality score which was validated against egg survival. The first multivariate egg quality score (PC1) differed for fish held in the tank and raceway systems. Egg survival, chorion breaking strength and Se chorion concentrations were higher in eggs produced by broodstock held in the S.C.E.N.E. system compared to those in the Ae system. In contrast, chorion concentrations of P and K were higher in eggs from fish held in the Ae system. These data highlight the complex interactions that exist between the holding environment and pre-ovulating fish and egg quality. Despite the importance of egg quality to the aquaculture industry, how the broodstock holding environment affects the eggs produced remains poorly understood.

3.2 Introduction

The quality of eggs produced by farmed fish continues to be a significant limiting factor in several branches of the aquaculture industry (Kjorsvik *et al.*, 1990). For example, species such as the Atlantic halibut (*Hippoglossus hippoglossus*) have considerable potential for commercial production; however hatching rates of less than 1% continue to obstruct the growth within the industry (Mommens *et al.*, 2010; Norberg *et al.*, 1991). Even in families such as Salmonidae, where there has been considerable work on culture and incubation systems, egg mortality rates of up to 50% still occur (Bobe and Labbe, 2010; Bromage *et al.*, 1992; Brooks *et al.*, 1997).

How egg quality is defined is vital in considering ways to improve these problems. In the salmonid aquaculture industry, egg quality is assessed by the number of eggs which fertilise, reach the eyed stage of development and hatch

successfully (Bobe and Labbe, 2010; Brooks *et al.*, 1997). However, while survival rates provide information on hatching success they provide no information on egg quality characteristics before and after fertilisation that may have influenced hatching success. Previous studies have suggested a number of parameters of the fish egg that may be used to determine egg quality; these include egg size, morphology, the distribution of yolk components within the egg and the biochemistry of the ovarian fluid (Bobe and Labbe, 2010; Brooks *et al.*, 1997; Kjorsvik *et al.*, 1990).

Studies have shown that there are a number of factors which may affect egg quality during various stages of its development (Bobe and Labbe, 2010). For instance, the quality of an egg may be influenced by the intrinsic properties of the brood fish prior to, and during ovulation (Brooks *et al.*, 1997). For example, female rainbow trout (*Oncorhynchus mykiss*) with elevated stress levels (as defined by increased levels of cortisol in the blood) 9 months prior to spawning produced eggs with significantly lower survival rates compared to fish with lower stress levels (Campbell *et al.*, 1992). Furthermore, a diverse selection of dietary components, including lipids, fatty acids, protein and trace minerals have also been shown to influence egg quality (Brooks *et al.*, 1997; Izquierdo *et al.*, 2001). For example, when fed a diet deficient in carbohydrate content, eggs produced by rainbow trout had a reduced survival rate to the eyed stage of egg development (Washburn *et al.*, 1990).

Once the egg is released, extrinsic factors such as the physiochemical conditions of the water (temperature, salinity and pH) in which the egg is fertilised and incubated will also affect egg quality (Brooks *et al.*, 1997). Water temperature during incubation is particularly important as it may affect metabolism, development and subsequently survival of the embryo (Kinne and Kinne, 1961). In salmonids, extremely high or low temperatures can also significantly impact egg mortality at early stages of development (Brooks *et al.*, 1997)

While many previous investigations have identified how intrinsic factors affect egg quality in pre-ovulating broodstock, little is known about how extrinsic factors, such as the holding environment affect broodstock in a way that impacts upon egg quality (Kjorsvik *et al.*, 1990; Brooks *et al.*, 1997; Izquierdo *et al.*, 2001). Therefore, the aim of the current study, is to examine how the

environment effects parameters of egg quality, mediated through the female by rearing two groups of brown trout, from the same strain, in two different rearing environments. In addition to utilising standard methods to define egg quality we also examined other novel measures of egg quality and how alternative data analysis can be used to test for differences in egg quality.

3.3 Materials and Methods

3.3.1 Samples and Location

Forty, 2+ female brown trout (*Salmo trutta*) from the Ae Fishery, Dumfries, were used in this study. All fish were from the same strain. Twenty trout were transported to aquarium facilities at the Scottish Centre of Ecology and the Natural Environment (S.C.E.N.E.), University of Glasgow, and held at these facilities from June 2008 until February 2009. The remaining 20 trout were held at Ae Fishery from June 2008 until November 2008.

3.3.2 Broodstock holding environment: S.C.E.N.E. system

Broodstock were held in an 800L round, polyethylene tank with a continuous supply of water (ca. 25L/min) from Loch Lomond, provided by three inlet pipes equidistant around the side of the tank. Waste water was drained using a central stand-pipe covered by a 5mm mesh screen. Fish were exposed to ambient Loch Lomond water temperature and photoperiod, delivered by artificial fluorescent lighting adjusted weekly to reflect natural photoperiod (Latitude =56.1403^{o}N). Industrial food pellets (EWOS Ltd) were dispensed daily into the tank by a clockwork belt feeder (Dryden Aqua Ltd) over a 24hr feeding period. The amount of feed dispensed daily, exceeded the recommended weight calculated from fish biomass and recommended feeding rate for salmonids. Water temperature for the duration of the experiment ranged from 6^{o}C to 15^{o}C (mean=11^{o}C).

3.3.3 Broodstock holding environment: Ae system

Trout were held in a 1000L raceway in a building at Ae Fishery. Both ends of the building were open sided and the roof comprised a transparent acrylic sheet, thus female broodstock were exposed to a natural photoperiod (latitude =55.1876^{o}N) with no artificial light sources. Water, drawn from the River Ae,

entered the raceway from a pipe at one end of the raceway (ca. 30L/min), while waste water was drained by a standpipe screened by 5mm mesh located at the other end of the raceway. Industrial food pellets (EWOS Ltd) were deposited into the tank by a clockwork belt feeder (Dryden Aqua Ltd) set to a 24hr continual feeding regime. The amount of feed dispensed daily, exceeded the recommended weight calculated from fish biomass and recommended feeding rate for salmonids. Water temperature tracked ambient for the River Ae, ranging from 1^{o}C to 20^{o}C (mean= 11^{o}C).

3.3.4 Assessing reproductive status of broodstock

Reproductive maturation in individual trout was assessed, between October 2008 and February 2009, by anaesthetising fish in a benzocaine solution (Sigma Life Sciences) and checking visually for signs of abdominal distension and egg release. Broodstock that were not ovulating were placed in a 150L recovery tank, before being returned to the holding tank. Ovulating fish were killed by exposure to a lethal dose of anaesthetic, followed by a sharp blow to the head (Schedule 1 method). Fish were blotted dry and their eggs were stripped into clean dry plastic tubs by abdominal manipulation.

3.3.5 Egg Survival

Approximately 500 eggs (estimated by weight) collected from 7 females at the Ae system and 10 females held at the S.C.E.N.E. system. Eggs from each female were sub-divided into two replicates, activated and placed into individual custom built incubation trays. At Ae Fishery, eggs were fertilised by male brown trout held there, at S.C.E.N.E., these were fertilised by a male of Howietoun fish farm origin. Eggs from fish reared at both Ae Fishery and S.C.E.N.E. were incubated at S.C.E.N.E.. Incubation trays were constructed from plastic mesh (5mm mesh diameter) wrapped round a solid square Perspex base and rim (10cmx10cmx15cm). These trays were placed in a 200L flow-through tank (5L min^{-1}) containing two water filters (Fluval A460). The eggs in individual trays were checked every alternate day for mortalities. Dead eggs were identified by their white/opaque appearance recorded and removed from the incubation system. The experiment was terminated when all surviving eggs reached the

eyed stage of development. Water temperature during egg incubation ranged between 5°C and 14°C (mean= 8°C)

3.3.6 Chorion breaking strength

Ten unfertilised, non-water hardened and ten unfertilised, water hardened eggs from each female were selected to measure chorion strength. The breaking strength of each individual chorion was tested using a Lloyd LRX compression test instrument (1mm diameter blunt ended probe, 5mm/min probe speed with a 5N load cell) (Ametek Inc).

3.3.7 Chorion element concentrations

Four unfertilised, non-water hardened eggs from each female were gently punctured with a sterilised needle. The yolk was extruded by gentle manipulation of the chorion. Each chorion was then placed in individually marked well-plates, wrapped in tinfoil and dried at 37°C for 24 hours. These were then mounted onto aluminium stubs using double sided sticky carbon tabs (Gisbourne Microscopy Services). Analysis of elemental concentrations was then carried out by energy-dispersive x-ray spectroscopy (E.D.X.), using a Philips XL30 ESEM equipped with a Phoenix energy dispersive x-ray detector (operating voltage = 20kv, working distance = 10mm) at the General Microscope Facility (University of Dundee). Carbon (C), oxygen (O), sodium (Na), magnesium (Mg), phosphorus (P), sulphur (S), chlorine (Cl), potassium (K), calcium (Ca) and selenium (Se) were all consistently detected during analysis. The percentage concentration of each of these elements was derived for the chorion of each individual egg.

3.3.8 Data analysis

Principle components analysis (PCA) was used to reduce the number of egg quality variables into a single multivariate egg quality score. To test the validity of using PCA scores in defining egg quality, PCA scores were regressed on egg survival for each female. One-way analysis of variance (ANOVA) was then used to examine the effects of broodstock holding environmental on this multivariate egg quality score. The effect of broodstock holding environment on individual egg quality variables was examined using ANOVA. All data presented as

percentages were arcsine transformed prior to analysis. The Minitab® was used to analyse the data.

3.4 Results

3.4.1 Multivariate egg quality score and egg survival

The first principle component (PC 1) of the PCA analysis of 12 putative egg quality variables, accounted for 37.8% of the total variance of the PCA analysis and showed high negative coefficients for non-hardened and hardened chorion breaking strengths, O and Se chorion concentrations, as opposed to high positive coefficients for of P, S and K chorion concentrations (Table 3-1). The second, third and fourth components (PC2, PC3 and PC4 respectively) also accounted for 22.3%, 14.6% and 12.0% of the total variance respectively. PC2 showed a high negative coefficient for C chorion concentrations and high positive coefficients for Cl and Ca chorion concentrations, while PC3 showed a high negative coefficient for O chorion concentrations and high positive coefficients for Se and Na chorion concentrations. The variables contributing to PC2, PC3 and PC4 are shown in Table 3-1.

PC1 scores were negatively correlated with egg survival for each female ($F_{[1,16]}$=9.97, r^2= 0.399, p=0.007), while PC3 scores were positively correlated to egg survival for each female ($F_{[1,16]=}$4.88, r^2=0.399, p=0.043). There was no significant correlation for PC2 ($F_{[1,16]=}$0.27, r^2=0.018, p=0.609) or PC4 ($F_{[1,16]=}$2.67, r^2=0.151, p=0.123). Therefore, PC2 and PC4 scores do not appear to reflect elements of egg quality that affect survival and thus were not considered further.

Table 3-1: PCA co-efficient applied to each variable measured and the total variation explained (%).

Variable	PC1	PC2	PC3	PC4
Non-water hardened chorion breaking strength	-0.38	0.12	0.17	-0.32
Water hardened chorion breaking strength	-0.36	0.02	0.28	-0.31
Se chorion conc	-0.21	0.15	0.54	0.30
C chorion conc	0.24	-0.46	0.17	0.06
O chorion conc	-0.35	0.04	-0.34	-0.02
Na chorion conc	-0.06	-0.03	0.61	0.24
Mg chorion conc	0.09	0.12	-0.15	0.68
P chorion conc	0.42	-0.02	0.09	-0.26
S chorion conc	0.33	0.25	0.15	-0.32
Cl chorion conc	0.07	0.59	0.07	-0.03
K chorion conc	0.44	0.03	0.12	-0.10
Ca chorion conc	0.10	0.58	-0.10	0.07
Variance explained (%)	31.8	22.3	14.6	12.0

3.4.2 Multivariate egg quality index (PC1 and PC3) and broodstock holding environment

PC1 egg quality scores were significantly lower in eggs from fish reared in the S.C.E.N.E. system compared to the Ae system ($F_{[1,16]}$= 18.95, r^2=0.558, p=0.001) (Figure 3-1), indicating that eggs from broodstock held in the S.C.E.N.E. system had higher chorion breaking strength (water hardened and non-water hardened) and chorion O concentrations and had lower P, S and K chorion concentrations. There was no difference in PC3 egg quality scores of eggs between holding sites ($F_{[1,16]}$= 3.06, r^2=0.169, p=0.101) (Figure 3-1).

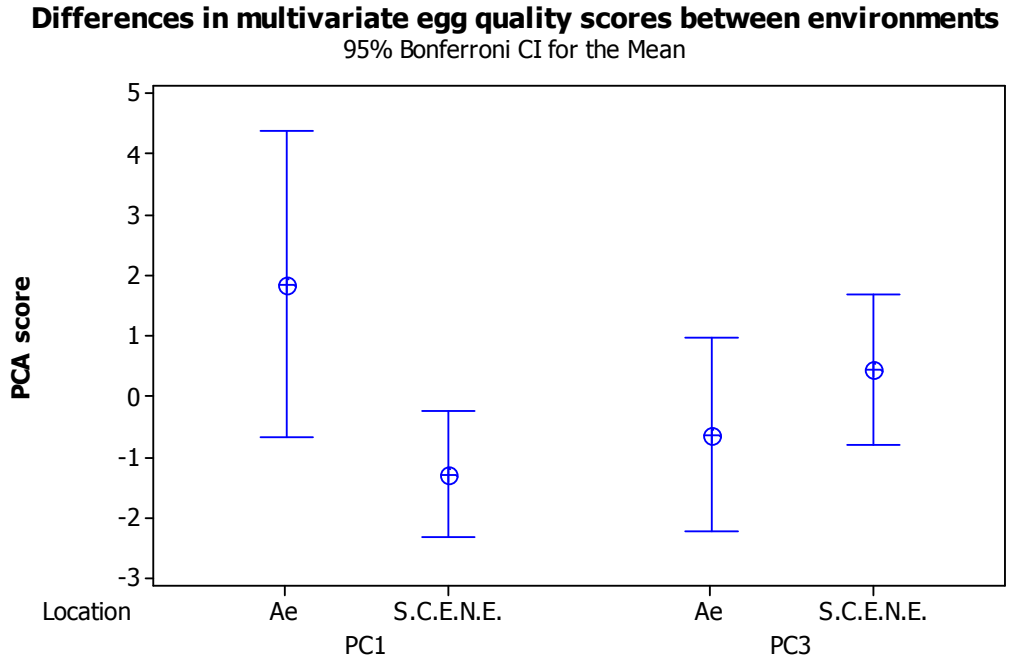

Figure 3-1: Differences in the egg quality score between environments. PC1, Ae (1.85±1.91) and S.C.E.N.E. systems (-1.29±1.07), and PC3, Ae (-0.63±1.21) and S.C.E.N.E. systems (0.44±1.27). (mean ± standard deviation).

3.4.3 The effect of broodstock holding environment on individual measures of egg quality

Eggs survival was significantly greater in eggs produced by females reared in the S.C.E.N.E. system compared to those held in the Ae system ($F_{[1,16]}$=31.8, r^2=0.66; p<0.001) (Table 3-2). Eggs produced by female broodstock held in the S.C.E.N.E. system also had a higher chorion breaking strength for both non-water hardened and water hardened eggs compared to those held in the Ae system (non-water hardened; $F_{[1,16]}$=201.3, r^2=0.93; p<0.001, water hardened; $F_{[1,16]}$=125.0, r^2=0.89; p<0.001) (Table 3-2).The chorion of the eggs produced by female broodstock held in the S.C.E.N.E. system had a higher percentage concentration of Se ($F_{[1,16]}$=5.1, r^2=0.20; P=0.040) (Table 3-2), whilst those from female broodstock held in the Ae system contained higher levels of both P and K (P; $F_{[1,16]}$=6.3, r^2=0.25; p=0.024) (K, $F_{[1,16]}$=11.5, r^2=0.40; p=0.004) (Table 3-2).

Table 3-2: The effect of broodstock holding environment on physical egg quality parameters and chorion element concentrations (Mn ± Std dev)

Variable	Ae	S.C.E.N.E.	P-value
Egg survival (%)	17.4±19.6	58.6±10.6	P<0.001
Non-water hardened chorion breaking strength (N)	0.7±0.6	6.8±1.0	P<0.001
Water hardened chorion breaking strength (N)	1.4±1.3	20.2±4.3	P<0.001
Se chorion conc. (%)	2.6±1.8	4.1±0.9	P=0.040
C chorion conc. (%)	48.8±1.8	47.7±2.7	P=0.369
O chorion conc. (%)	38.4±2.5	39.5±2.1	P=0.340
Na chorion conc. (%)	4.3±0.7	4.5±0.8	P=0.677
Mg chorion conc. (%)	5.6±4.6	3.7±0.7	P=0.213
P chorion conc. (%)	5.1±1.7	3.7±0.6	P=0.024
S chorion conc. (%)	4.9±1.8	4.1±1.6	P=0.347
Cl chorion conc. (%)	3.4±1.1	3.6±4.2	P=0.918
K chorion conc. (%)	4.1±1.4	2.2±1.0	P=0.004
Ca chorion conc. (%)	3.7±1.5	3.0±3.2	P=0.598

3.5 Discussion

The results show that the first principle component score explained 40% of variation in egg survival and thus was a good index of egg quality. The relationship between the scores of PC1 and egg survival shows the benefits of using integrated methods of analysis where a number of independent variables can be combined into a single, usable measure of egg quality. The results indicate that brown trout reared in S.C.E.N.E. system produced eggs of significantly higher quality compared to eggs produced by brown trout held in the Ae system. Previous studies have successfully used similar analyses to examine the effects of stocking density, disturbance and aggressive behaviour on multiple determinants of welfare in Atlantic salmon (Adams *et al.*, 2007; Turnbull *et al.*, 2005).

However other factors not measured during this experiment may also have had an effect. For example, Aegerter *et al.*, (2005) reported differences in the presence of specific mRNA transcripts, between high and low quality eggs, while Arukwe and Goksoyr, (2003) reported that low levels of yolk proteins stored within the egg prior to ovulation might affect embryogenesis and thus egg survival. Therefore, further studies integrating both chorion egg quality parameters and yolk egg quality parameters into a single measure of egg quality

may explain more of the variation in egg survival than was shown within the current study.

Broodstock reared in S.C.E.N.E. and Ae systems produced eggs which differed in egg survival, chorion breaking strength (non water hardened and water hardened) and chorion Se concentrations. This strongly indicates that broodstock rearing environment modified these parameters thus increasing egg survival, chorion breaking strength and chorion Se concentrations eggs from trout held in the system at S.C.E.N.E. or decreasing the same parameters in eggs from trout held at Ae Fishery.

The chorion breaking strength measurements for eggs obtained from S.C.E.N.E. were comparable to similar studies on other salmonids, however the measurements obtained for eggs produced by broodstock held at Ae Fishery were distinctly lower (Iuchi *et al.*, 1996; Zotin 1958). For example, eggs from rainbow trout ruptured under a force of 29N, 24 hours after being water hardened (Iuchi *et al.*, 1996). This suggests that the Ae system caused trout to produce eggs with a reduced chorion breaking strength rather than the system at S.C.E.N.E. causing trout to produce eggs with increased chorion breaking strength. The chorion is a proteinaceous membrane which surrounds the egg and protects the embryo inside from mechanical and chemical stressors during development (Kudo 1992; Yamagami *et al.*, 1992). Aquaculture procedures such as broodstock stripping and egg sorting can cause mechanical damage to the eggs due to the physical nature of the process, however, eggs with a more robust chorion are more likely to be able survive stripping and sorting process intact and undamaged.

Selenium plays a pivotal role against oxidative cellular injury (Rider *et al.*, 2009). Of the 30 or so selenoproteins identified in mammals, a similar number of homologues have been recognised in fish (Kryukov and Gladyshev, 2000). The most studied of these are the enzymes glutathione peroxidase (GSH-Px), thioredoxin reductase (TR), catalase and superoxide dismutase are important molecules involved in intracellular antioxidant defence (Arthur 2000; Halliwell 1999; Arteel and Sies, 2001). All broodstock diets contain some form of Se, which is usually stored and metabolised within the liver. This organ is also where the chorion proteins are manufactured before being transported to the ovary

and overlaid onto the developing oocyte (Arukwe and Goksoyr, 2003; Rider *et al.*, 2010). The increased concentrations of chorionic Se may indicate that eggs produced by broodstock held within the Ae system were better protected from oxidative stress compared to eggs produced by raceway broodstock.

Chorion concentrations of P and K were significantly higher in eggs produced by broodstock held in the Ae compared to the S.C.E.N.E. system. The elevated chorion concentrations of P and K may indicate poor egg development prior to and/or during ovulation. Craik and Harvey (1984) observed that, protein linked phosphorus levels were higher during unsuccessful oocyte hydration compared to when oocytes successfully hydrated. Potassium ions are also essential during volume increase and water uptake of maturing oocytes although elevated levels of K are indicative of osmoregulatory failure in salmonids (Bjornsson *et al.*, 1989; Cardeilhac *et al.*, 1979; Greeley *et al.*, 1991; Redding and Schreck, 1983; Liebert and Schreck, 2006). Phospholipids, inorganic phosphate and potassium are universally major components of living cells (Craik 1982; Craik and Harvey, 1984; 1986; Lafleur and Thomas 1991), however in terms of their function within the chorion; this is most likely to be associated with egg hydration.

There were other differences between sites which may have impacted on egg quality. Logistical difficulties meant that eggs produced at Ae Fishery were fertilised before being transported to S.C.E.N.E. for incubation and it is possible that this may account for the significantly lower survival rate, however the measurable differences in other egg quality parameters indicate that the transport process was less likely to have had an effect. Although day length was the same at both Ae Fishery and S.C.E.N.E., the photoperiod at S.C.E.N.E. was artificial compared to the natural photoperiod at Ae Fishery. The effect of photoperiod on egg quality is complicated for instance, age and body size of broodstock, water temperature, and length of oogenesis can differ from variations in photoperiod (Bode and Labbe, 2010). Together with factors such as, temperature, husbandry, stress and hydrology it is thus possible that these factors rather than the holding facilities influenced the results reported above.

In conclusion the results of both the integrated measures of chorion quality, individual chorion quality parameters and egg survival rates suggest the rearing environment had an effect on egg quality mediated through the broodstock. Eggs

produced by brown trout reared in the S.C.E.N.E. system were of significantly higher quality compared to eggs produced by trout reared within the Ae system.

4 Intra-population variation in egg chorion quality in Atlantic salmon (*Salmo salar*)

4.1 Abstract

Variable egg survival rates are currently one of the major factors limiting expansion of the aquaculture industry. During the current study the variation in egg survival in individual Atlantic salmon reared in the same environment was assessed and used to examine the suitability of 4 measures of the chorion (chorion elemental concentrations, chorion breaking strength and chorion protein profile and concentrations) as parameters of egg quality. There was a significant variation in egg survival between individual salmon. There was also a large amount of variation in egg survival, chorion breaking strength, chorion elemental concentrations and chorion protein concentrations and profiles between individual Atlantic salmon (*Salmo salar*). Subsequent analysis of the data showed that there was no difference in these chorion quality parameters between high and low egg survival rates. Furthermore, there was no correlation between egg survival and the chorion quality parameters recorded during this study. These results suggest that either chorion quality parameters not examined during this study or alternative components of the egg, such as the yolk, were responsible for the significant variation in egg survival. The results also show that individual variation between fish is an important factor affecting egg quality.

4.2 Introduction

Variation in egg survival rates from individual fish are well documented in several cultured marine and freshwater species including Atlantic cod (*Gadus morhua*), turbot (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglossus*), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) (Bromage *et al.*,1992; Kjorsvik *et al.*, 1990; Kjorsvik *et al.*, 2003). Briggs (1953) first reported that salmonid egg survival varied between 0% and 85% in various hatcheries across the United States of America. Some 30 years later and despite extensive studies and significant improvements in aquaculture methods this variation is still affecting the growth of the industry (Springate and Bromage 1983; 1984a; 1984b; Bromage and Cumaranatunga, 1988)

Complicating this issue is that apart from egg survival, there is no consistently reproducible egg quality parameter which can be used to identify high or low quality in eggs from cultured fish (Bode and Labbe, 2011). Most studies investigating egg quality also choose to focus on only one measure of egg quality and how this relates to egg survival, this approach fails to take account of any complex interactions between variables.

During oocyte maturation the female provisions the immature egg cells with minerals and nutrients needed to construct the chorion, these are essential in allowing the embryo to continue to develop normally and survive onto hatching and first feeding (Brooks *et al.*, 1997). For example the chorion functions as an interface between the egg and sperm during fertilisation and as barrier between the egg and its environment during embryogenesis (Arukwe and Goksoyr, 2003). Despite these important functions, little is known about the relationship between the chorion and egg quality, i.e. which measures of the chorion are likely to predict egg quality (Bode and Labbe, 2010; Brooks *et al.*, 1997).

Results from the previous chapter (Chapter 3) found a relationship between an integrated index of chorion quality parameters and egg survival in brown trout. Significant differences in chorion egg quality parameters between brown trout, held in two different rearing environments were also observed during this study. Here I focus on the individual variation in egg quality displayed by the more commercially important species, Atlantic salmon, which had been reared under the same extrinsic conditions. Therefore, the aim of this study was to compare chorion quality parameters and examine their relationship with egg survival in Atlantic salmon while investigating the level of variation in chorion quality characteristics by fish which had been reared in a homogenous environment.

4.3 Materials and Methods

4.3.1 Samples and location

Forty, 1 sea-winter female Atlantic salmon from Landcatch Ltd Ormsary aquaculture facility were used in this study. All fish were from the same strain. Salmon broodstock used comprised farm origin fish which bred in the winter

05/06 then raised at Landcatch Ltd Ormsary until they themselves spawned during winter 09.

4.3.2 Broodstock holding facilities

Five hundred fish were held in 200m^3 round concrete ground tank at Ormsary. A continuous supply of water (ca. 45L/sec) from Loch Caolisport was added via inlet pipes, at the side of the tank. Waste water was drained using a pipe located in the centre of the tank. A meshed metal grate (10cm mesh diameter) prevented stock escapes. Fish were exposed to ambient Loch Caolisport water temperatures and Ormsary photoperiod (Latitude =55.8879^{o}N). Fish in the broodstock tank were hand fed a recommended amount of industrial food pellets (EWOS Ltd) depending on biomass and ambient temperature. Food pellets were dispensed daily by hand. Water temperature for the duration of the experiment ranged from 6.1^{o}C to 16.6^{o}C (mean=10.9^{o}C).

4.3.3 Assessing reproductive status of broodstock

Broodstock were herded into an isolated 50m^3 section of the tank. This section was isolated using a 6x 1m frame with a net mesh and a tarpaulin sheet to allow for anaesthesia of the fish. Reproductive maturation in individual salmon was assessed by anaesthetising fish within the 50m^3 section with a benzocaine solution (Sigma Life Sciences) and manually checking for signs of abdominal distension and egg release. Broodstock that were not ovulating were returned to the main section of the tank. Ovulating fish were placed in a 0.75m^3 container and transferred to one of four 1m^3 fibreglass holding tanks. Here they were given a lethal dose of anaesthetic, blotted dry and their eggs were stripped into clean dry plastic 10L buckets by inserting a nozzle into the oviduct and releasing compressed air.

4.3.4 Egg survival

Eggs collected from each female were activated by fertilisation, via a single male, and water hardened before being placed into individual custom-built incubation containers. Incubation containers were constructed from 10L plastic buckets. An outflow standpipe was located in the centre of the container and a raised platform of metal mesh (5mm in diameter) encouraged water to circulate

at the bottom of the incubation system. The water fed from Loch Caolisport to the Ormsary Medium Header tank was treated with ozone (Sterner Aquatech) before reaching the incubation system. After treatment the water was filtered into each incubation container by a lid which had ten 10mm holes drilled into it. Once eggs had reached the eyed stage of development, they were auto-sorted using an Impex egg sorter. Dead eggs were identified by their white/opaque appearance and removed from the incubation system. Numbers of mortalities were recorded for eggs from each female. Egg survival was defined as the number of live eggs returned to the incubation system after the auto-sorting process. Water temperature during egg incubation ranged between 1.4^{o}C and 7.7^{o}C (mean= 3.6^{o}C).

4.3.5 Chorion breaking strength

The details of how chorion breaking strength was measured can be found in Chapter 2. In brief, 10 eyed and ten un-eyed eggs were collected from each individual female and the breaking strength tested using a Lloyd LRX compression test instrument.

4.3.6 Chorion element concentrations

The details of how chorion element concentrations were measured can be found in Chapter 2. In brief, chorions from 4 un-eyed eggs, taken from individual salmon classified as producing eggs with high or low survival rates, were removed then oven dried and mounted onto sticky tabs. Analysis of the chorion elemental concentrations was carried out by energy-dispersive x-ray spectroscopy and nitrogen (N), zinc (Zn), magnesium (Mg), phosphorus (P), sulphur (S), iron (Fe), potassium (K), calcium (Ca) and selenium (Se) were all consistently detected during analysis.

4.3.7 Protein assay and SDS-gel electrophoresis of the chorion

The details of how the chorion was processed for SDS gel electrophoresis can be found in Chapter 2 with the following amendments. Ten chorions from un-eyed eggs and from individual female salmon categorised as producing eggs with high and low survival rates, were washed in pH 7.8, 0.2M Tris buffer for 30mins with

three consecutive changes of buffer before being macerated in homogenisation buffer.

Due to the small amounts of protein present, chorion samples were subjected to an acetone precipitation. Approximately 1ml of acetone was added to an eppendorf containing 250µl of sample, and stored at -20^{o}C overnight. Samples were centrifuged at 13,000rpm for 5 minutes at 4^{o}C, forming a pellet at the bottom of the eppendorf. Excess acetone was removed via pipetting and vortex drying at 40^{o}C (Christ RVC 2-25. The pellet was re-suspending in 100µl of chilled 0.01M phosphate buffered solution (PBS), and dissolved using pipette and vortex agitation before undergoing sonication (1 sec pulse, amplitude= 40) to ensure that it had completely dissolved.

The chorion protein concentration was then measured using a modified version of the Lowry assay (Lowry *et al.*, 1951) after which, 5µg/ml of protein chorion samples were prepared and run for protein profiling via SDS gel electrophoresis. Once electrophoresis was complete gels were processed using SilverQuest™ (Invitrogen), and all clearly defined protein bands were scanned into the computer for image analysis using Scion imaging that calculated the pixel density of the selected bands in question. In this case pixel density data was used as a semi quantitative measurement of protein concentrations.

4.3.8 Data analysis

All data presented as percentages were arcsine transformed before statistical analysis. One-way analysis of variance (ANOVA) was used to examine the differences in egg survival, chorion burst strength, chorion elemental concentrations and chorion protein concentrations between the female Atlantic salmon which produced eggs with high and low survival rates. Linear regression was used to assess the relationship between egg survival and chorion elemental concentrations, chorion breaking strength and chorion protein concentrations. The Minitab® 16 was used for data analysis.

4.4 Results

Of the 40 egg groups 12 recorded 100% mortality before reaching the eyed stage. Eggs from these families were incubated in close proximity to one another, suggesting a catastrophic failure in survival may have been caused by a localised problem in the incubation system and not an egg quality issue. These egg groups were not included in further data analysis.

4.4.1 Variation in egg survival between individual Atlantic salmon

Of the remaining groups there was a great deal of variation in egg survival rates. The mean survival rate of eggs from Atlantic salmon obtained during this study was 78%±15% (mean ± standard deviation) and ranged from 44-99%.

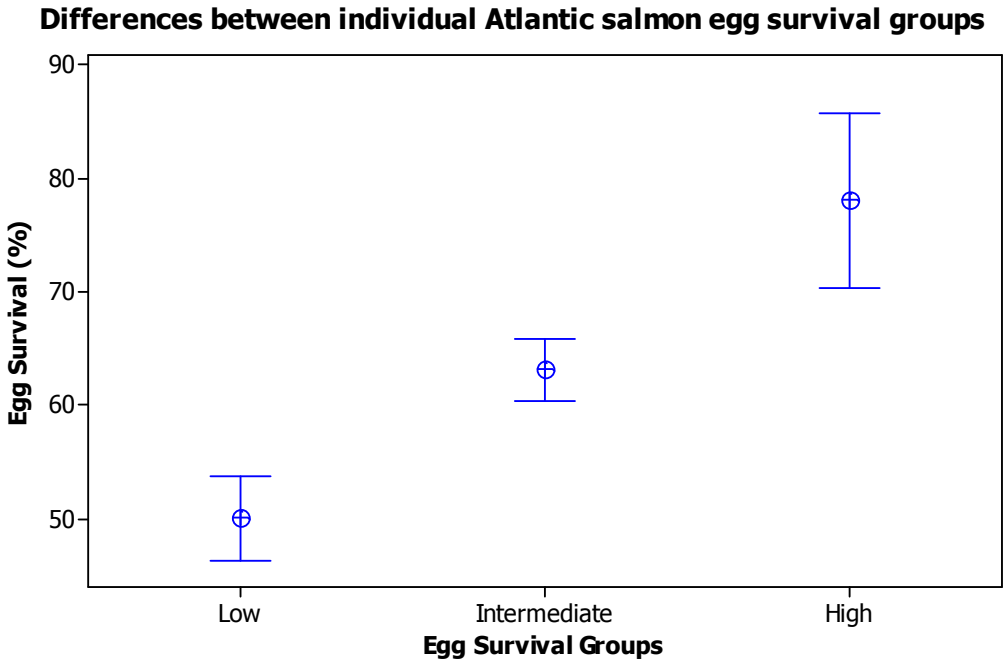

Figure 4-1: Differences between individual Atlantic salmon egg survival groups. Interval plot showing the difference in egg survival between high (78.1±10.0), intermediate (63.2±4.1) and low (50.0±4.5) performing families (mean ± standard deviation).

To examine differences between egg groups exhibiting high/low survival rates, groups were categorised as either low (<60%), intermediate (60-70%) or high (>70%). Not surprisingly there was a significant difference in egg survival between eggs produced by Atlantic salmon with high survival rates and low survival rates ($F_{[2,27]}$= 37.7; r^2 =75.1, p< 0.01) (Figure 4-1). Further analysis of

chorion quality parameters focused on the chorions of eggs from individual females with the highest and lowest egg survival rates as these are 1) the most commercially important groups and 2) more likely to provide significant differences in the chorion quality parameters examined.

4.4.2 Variation in chorion quality parameters between eggs from females exhibiting high or low egg survival rates

There was no significant difference in chorion breaking strength or chorion elemental analysis between fish producing eggs with high survival rates and those with low survival rates (Table 4-1), although there is variation in the means within egg survival groups for both chorion breaking strength and chorion elemental analysis (Table 4-1)

Table 4-1: Variation in chorion quality parameters from individual Atlantic salmon categorised as either high and low egg survival groups.

Factor	High Egg survival (Mn ± Std dev)	Low egg survival (Mn ± Std dev)	P-value
Un-eyed chorion breaking strength(N)	1.49±0.17	1.44±0.13	0.491
Eyed chorion breaking strength(N)	1.79±0.20	1.68±0.29	0.422
Ca chorion conc(%).	5.54±1.74	5.21±2.76	0.775
Fe chorion conc(%).	9.61±5.14	14.27±10.70	0.287
K chorion conc(%).	5.82±1.23	5.58±1.64	0.754
Mg chorion conc(%).	10.25±2.45	8.70±3.65	0.336
N chorion conc(%).	56.04±16.49	46.29±18.39	0.282
P chorion conc(%).	12.91±4.11	10.51±4.55	0.286
S chorion conc(%).	11.65±4.76	10.79±5.66	0.748
Se chorion conc(%).	4.38±4.11	7.23±6.35	0.546
Zn chorion conc(%).	4.09±2.82	5.19±4.13	0.305

4.4.3 Relationship between chorion egg quality characteristics and egg survival.

There was no significant linear relationship between chorion breaking strength of eyed or un-eyed eggs and survival. There was also no significant linear relationship between chorion elemental concentrations from un-eyed eggs and egg survival (Table 4-2).

Table 4-2: Chorion quality parameters regressed on egg survival to examine linear relationships.

Factor v Egg survival	P-value
Un-eyed chorion breaking strength(N)	0.763
Eyed chorion breaking strength(N)	0.386
Ca chorion conc(%).	0.620
Fe chorion conc(%).	0.333
K chorion conc(%).	0.700
Mg chorion conc(%).	0.276
N chorion conc(%).	0.119
P chorion conc(%).	0.406
S chorion conc(%).	0.862
Se chorion conc(%).	0.342
Zn chorion conc(%).	0.575

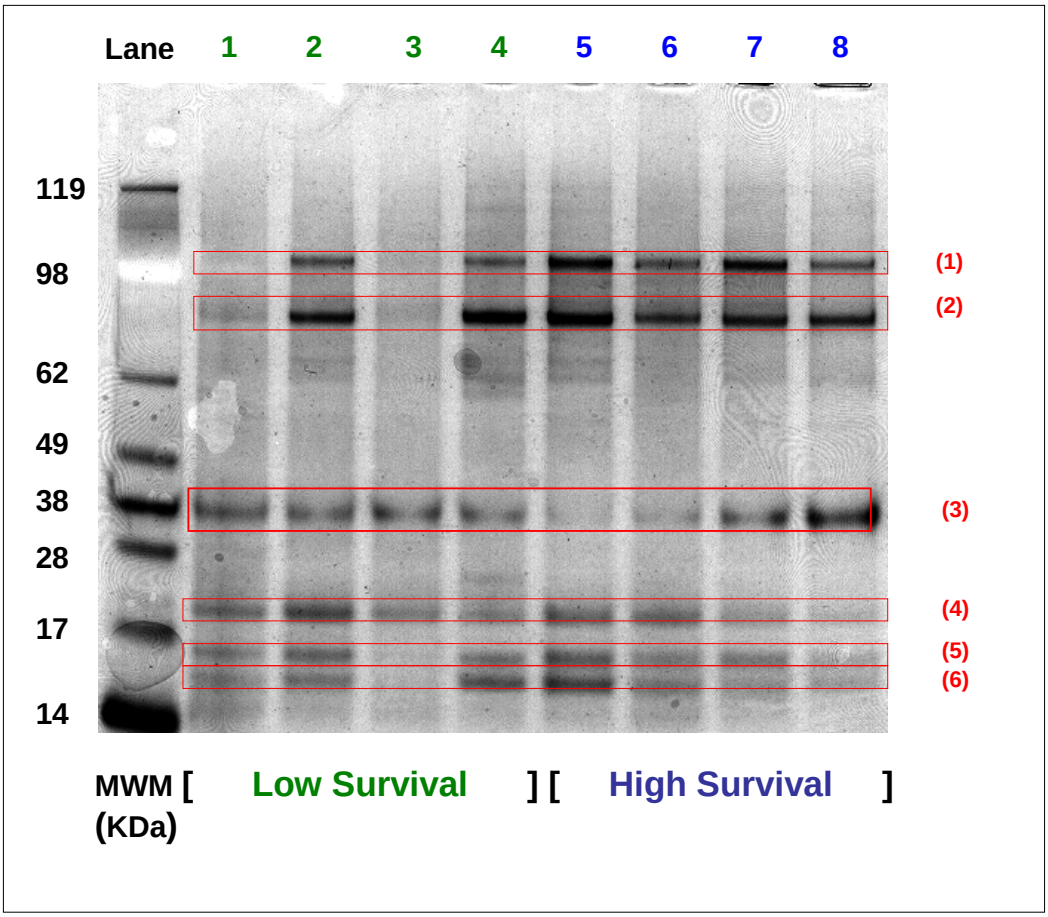

Figure 4-2: Gel containing chorion protein profiles from a selection of Atlantic salmon. Lanes 1-4 contain chorion protein profiles from eggs produced by individuals categorised as having low survival rates while lanes 5-8 contain chorion protein profiles from eggs produced by individuals categorised as having high survival rates. The 6 bands containing proteins, selected due to their clearly defined profile are shown within the red boxes.

4.4.4 Variation in chorion protein profiles and concentrations in eggs families with high and low survival rates

There was no significant difference in the protein profile or concentration of Atlantic salmon chorions from eggs with high or low egg survival rates (Table 4-3). Figure 4-2 shows the 6 bands selected for protein analysis (approximately 99KDa, 80KDa, 38KDa, 20KDa, 16KDa and 15KDa) and provides an example of the variability of chorion protein concentrations within this population of Atlantic salmon. Pixel density analysis confirms the large variation in chorion protein concentrations (Table 4-3).

Table 4-3: Differences in protein concentrations (expressed as pixel density data) between chorions from Atlantic salmon from high and low egg survival families.

Protein Band	High egg survival family protein conc. (Mn ± Std dev)	Low egg survival family protein conc. (Mn ± std dev)	P-value
1	7981 ± 1898	5732 ± 2619	0.069
2	8707 ± 2264	6389 ± 3149	0.113
3	5626 ± 1285	5942 ± 937	0.583
4	5587 ± 3323	6044 ± 4329	0.816
5	3629 ± 2693	3380 ± 3012	0.864
6	3682 ± 3329	3508 ± 3229	0.917

4.5 Discussion

The results show variation in chorion quality parameters between individual females as well as egg survival groups, however, there was no significant difference in chorion breaking strength, elemental composition or protein profiles and concentrations between eggs from Atlantic salmon categorised as having high or low survival rates. There was also no significant linear relationship between chorion breaking strength or elemental composition and egg survival from the same individuals. Chapter 3 showed the importance of the chorion breaking strength and elemental composition in relation to egg survival for brown trout, however, there was no evidence of this in Atlantic salmon from Landcatch Ormsary in this study.

The chorion is a highly conserved structure within teleosts, although some species specific differences do exist (Berois *et al.*, 2007). Comparisons of the current study chorion protein profile with previous studies is inadvisable due to differences in protein profiles which may arise between families, species, stage

of egg development, preparation of samples and the affinity of the stain used. For example, Arukwe and Goksoyr, (2003) reported that there are between 3 and 4 proteins which consistently occur during electrophoresis in salmonid eggs, however, while two separate studies on the chorion protein profile of rainbow trout (*Oncorhynchus mykiss*) eggs each identified 4 proteins, they were at different molecular weights (Brivio *et al.*, 1991; Iuchi *et al.*, 1996). Whether or not these inconsistencies were due to population differences or differences in the preparation of samples is not known, but it does highlight the importance of consistency when performing molecular biological techniques. Therefore, this study has provided a standard protocol, which will be used to process chorions from eggs produced by Atlantic salmon fed non-supplemented and supplemented diets in Chapter 5.

The unfertilised and non-water hardened eggs of teleosts are generally composed of the following components, the micropyle, a nucleated blastodisc, a vitelline membrane, yolk and chorion (Brooks *et al.*, 1997). These components function synergistically and contribute to egg survival and embryonic development. The micropyle, nucleated blastodisc and vitelline membrane are components associated with successful fertilisation of eggs. During the current study, egg survival was calculated from eggs which had fertilised successfully and developed onto the eyed stage of development, therefore it is unlikely that these components contributed to the observed variation in egg survival and thus this leaves only the chorion and yolk contents as possible contributors.

The chorion is a complex proteinaceous membrane which is both robust and flexible. Simultaneously, it prevents damage to the embryo by functioning as a mechanical and biochemical barrier, while also facilitating gas exchange for respiration by diffusion (Arukwe and Goksoyr, 2005). This study focused on one specific component of egg quality, the chorion, and did not find a significant relationship between the tested parameters, however, chorion parameters not investigated during this study may have contributed to the observed variations in egg survival. For example, the permeability of the chorion and its ability to transport oxygen to the embryo has been shown to affect egg survival. If oxygen transfer across the chorion for respiration declines, this can result in growth deficiencies and mortalities (Greig *et al.*, 2005)

The chorion is the outermost component of the egg and encapsulates a rich fluid filled centre, which is often referred to as the 'yolk', however, this substance contains a variety of components which are essential if the embryo is to continue to develop. The yolk contains, amongst other molecules, proteins and mRNA transcripts. During egg development maternally derived mRNA accumulates within the immature egg cell (Tata, 1986; Howley and Ho, 2000; Pelegri 2003). After fertilisation these maternally derived molecules support early embryonic development until the initiation of zygotic transcription, which occurs during the Maternal-Embryo Transfer (MET) or mid-blastula stage of embryogenesis (Kane and Kimmel, 1993). Aegerter *et al.*, (2005) observed different concentrations of seven mRNA transcripts between low quality eggs and high quality eggs produced by rainbow trout (*Oncorhynchus mykiss*). During a similar study by Bonnet *et al.*, (2007) it was reported that the abundance of prohibitin 2 (PHB 2) within the eggs of rainbow trout, a molecule involved in regulating the endocrine system and mitochondrial respiration in mammals, was negatively correlated with egg survival.

When reared within the same environment, differences between individual fish at the genetic level are likely to strongly influence egg quality (Bode and Labbe, 2010). Preliminary studies on genetic influences on fish egg quality indicate that within a single population of rainbow trout, females that produce high quality eggs during their first spawning year do so again in the subsequent year, suggesting that there are genetic influences on egg quality (Brooks *et al.*, 1997). However, due to zygotic transcription replacing maternally derived systems during the mid-blastula transition, the genetic influence of maternal genes is difficult to assess in fish (Bode and Labbe, 2010).

In conclusion these results suggest that chorion quality parameters, specifically chorion breaking strength, chorion elemental concentrations and chorion protein abundances are not adequate indicators of egg quality in Atlantic salmon due to the lack of a relationship between these parameters and egg survival. Parameters such as yolk content and/or alternative chorion parameters may have contributed to the observed variations in egg survival. However, while extrinsic factors may affect egg quality in populations reared in two different environments (Chapter 3), variations in the maternal genetics between

individuals may be more predictive of egg quality in fish reared within a homogenous environment.

5 Maternal transfer of selenium to the eggs of Atlantic salmon (*Salmo salar*) fed a supplemented diet and the effect on egg quality parameters

5.1 Abstract

Broodstock Atlantic salmon (*Salmo salar*) were fed a standard commercial diet, with or without the addition of a supplemented nutritional mix, which included higher concentrations of the trace mineral selenium (Se). After spawning, the Se content of the eggs and livers of each fish were assessed. Egg survival rates and proteomic analysis of the egg chorion was also carried out. Concentrations of Se in the eggs of the individuals fed the supplemented diet were significantly higher than those fed the non-supplemented diet. However the survival rate of eggs from females given a supplemented diet was significantly lower. The assessment of the chorion protein profile and its proteomic structure was inconclusive. Nevertheless, the results support the hypothesis that female broodstock receiving a Se supplemented diet transfer this trace mineral into their eggs. The lack of a correlation between Se egg concentrations and egg survival suggests that the lower egg survivorship of eggs from the broodstock fed the supplemented diet in this trial was due to another nutritional component of the diet rather than their Se content.

5.2 Introduction

Broodstock nutrition remains one of the most poorly understood and researched areas of aquaculture. As with humans and other mammals it is clear that dietary nutrient requirements of broodstock fish will be different from that of rapidly growing juveniles (Izquierdo *et al.*, 2001). Moreover, it appears that different fish species may have different dietary requirements at different stages of their lifecycle and consequently, diets for broodstock need to be adapted to suit these particular factors in order to ensure good egg quality (Izquierdo *et al.*, 2001). Over the past two decades nutritional research into the bulk dietary components i.e., proteins, fats and carbohydrates, and their effect on egg quality has received more attention than so-called ‘minor’ dietary constituents, such as the trace elements zinc (Zn) and selenium (Se) and vitamins C and E

(Brooks *et al.*, 1997). However, even these 'minor' dietary components are involved in vital biological processes including skeleton formation, maintenance of the colloidal systems, regulation of acid-base equilibrium and the function of biologically active compounds including hormones and enzymes (Watanabe *et al.*, 1997). Furthermore, some studies have identified links between dietary trace element content and egg quality (Bobe and Labbe, 2010; Brooks *et al.*, 1997; Hardy 1983; Takeuchi *et al.*, 1981). Despite the importance of trace elements in fish nutrition and health, knowledge of their biological roles during reproduction under cultured conditions is limited.

Due to the importance of Se in metabolic processes and its role as an essential nutrient for a number of domestic mammals and birds, there is considerable interest among the aquaculture industry in the potential health benefits of Se in fish (Rider *et al.*, 2009). Indeed research on several marine and anadromous species such as salmonids has indicated that the health of farmed fish may be compromised by poor provisioning of trace elements such as Se within the diet. For example, Thorarinsson *et al.*, (1994) found that, when fed high concentrations of Se, mortality rates in Chinnock salmon (*Oncorhynchus tshawytscha*) infected with bacterial kidney disease fell significantly. In addition, increasing concentrations of Se within the diet significantly lowered the risk of liver lipid peroxidation, in juvenile rainbow trout (Rider *et al.*, 2009). Despite investigations such as these, many questions concerning the role of Se in nutrition remain unanswered. Of particular importance is whether or not broodstock receiving a Se supplemented diet transfer Se to their oocytes during maturation and what affect this may have on the egg survival during embryonic development. Answers to these questions may dramatically affect the development of broodstock nutrition within the aquaculture industry.

The fish egg is a complex biological structure consisting of six main components; chorion, yolk, micropyle, nucleated blastodisc, vitelline membrane and perivitelline space. The nutrients and minerals required to construct each of these components are maternally derived and require the female broodstock to be fed a nutritionally balanced diet (Bromage *et al.*, 1992). Each egg component is vital, either directly and/or indirectly, to one or more stages of larval development. For example, while the chorion is not directly involved in embryonic development, this tough outer membrane has several physiological

functions that are important for both fertilisation of the oocyte and protection of the growing embryo (Modig *et al.*, 2007). The chorion forms the first line of defence for the developing egg from the external environments after ovulation. It also plays an essential role in permitting sperm entry at fertilisation and in gas exchange during subsequent embryonic development (Arukwe and Goksoyr, 2003; Grierson and Neville, 1981).

While the function of the chorion has been thoroughly investigated, there is limited information concerning its relationship to egg quality or what effect dietary supplementation might have on the protein composition of this particular component of the egg. Therefore, the aim of this study is to investigate the effect of dietary supplementation of Atlantic salmon broodstock on fish egg quality with particular emphasis on the maternal transfer of Se and its effects on the protein composition of the chorion.

5.3 Materials and Methods

5.3.1 Samples and location

One sea-winter male and female Atlantic salmon (*Salmo salar*) obtained from Landcatch Ltd Ormsary aquaculture facility were used in this study. All fish were from the same strain. Salmon broodstock were spawned in winter 05/06, hatched and raised at Landcatch Ltd Ormsary until they themselves spawned during autumn/winter 2010/11.

5.3.2 Holding facilities for broodstock fed the non-supplemented diet (control condition)

One hundred and twenty five broodstock were placed into a 200m^3 round concrete ground tanks at Ormsary, a continuous supply of oxygenated water (ca. 30L/second) from Loch Caolisport was used. Waste water was drained via a central drain, covered with mesh to prevent escapes. Fish were exposed to ambient Loch Caolisport water temperatures and Ormsary photoperiod (Latitude =55.8879^{o}N). Fish were fed broodstock food pellets (EWOS Ltd), which were dispensed by hand. The volume of feed given to the fish was dependent on biomass and ambient water temperatures. The seawater temperature for the duration of the experiment ranged from 6.1^{o}C to 16.6^{o}C (mean= 10.9^{o}C).

5.3.3 Holding facilities for broodstock fed the supplemented diet (experimental condition)

Broodstock selected for dietary supplementation were held in four 200m^3 round concrete ground tanks at Ormsary. Each tank held 500 individuals. A continuous supply of oxygenated water (ca. 30L/sec) from Loch Caolisport was supplied. Waste water was drained via a central drain, covered with a mesh net to prevent escapes. Fish were exposed to ambient Loch Caolisport water temperatures and Ormsary photoperiod (Latitude =55.8879^oN). Broodstock were fed food pellets supplemented with Skretting Ltd, "Vitalis" mix, which were dispensed by hand. The Vitalis supplement was applied as a coating to the basal diet and consisted of an additional 0.5mg/kg of the organic Se compound, Sel-plex. The volume of feed given to the fish was dependent on biomass and ambient water temperatures. The seawater temperature for the duration of the experiment ranged from 6.1 ^oC to 16.6^oC (mean= 10.9^oC).

5.3.4 Assessing the reproductive status of the broodstock and sample collection

Prior to their spawning period, all feeding was discontinued and all broodstock (control and experimental) were transferred into a freshwater 1000m^3 round concrete ground tanks. Although the control and experimental populations were mixed, the tanks individuals were held in and the diet they received could still be identified as fish were individually tagged with radio frequency identification (RFID) chips. During the spawning period, fish were herded into an isolated 50m^3 section of the tank. This section of the tank was isolated using a crowder frame with a mesh net, and a tarpaulin sheet to allow for anaesthetising. Reproductive maturation in individuals was assessed by anaesthetising the fish within the isolated section with a benzocaine solution (Sigma Life Sciences) and manually checking for signs of abdominal distension and egg release. Broodstock that were not ovulating were released back into the main tank area. Ovulating fish were placed in a 0.75m^3 container and transferred to one of four 2m^3 fibreglass holding tanks. Here they were given a lethal dose of anaesthetic, blotted dry and their eggs were stripped into clean dry plastic 10L buckets using compressed air inserted into the vent in order to stimulate egg release from the coelomic cavity. Each bucket containing the eggs was then labelled with the identification number of the female from which the eggs had been harvested. A subsample of

eggs from 10 females which had been fed the control diet and 10 females in each of the four tanks fed the supplemented diet were selected for further assessment. The livers from each of these females was also harvested, placed into a sealed plastic bag and stored at -20^{o}C.

Males were separated from females using the crowder frame and given an intraperitoneal injection containing gonadotrophin releasing hormone (GnRH) to induce sperm production one week before females were checked for sexual maturation. The males were then identified using the RFID chip at the same time females were being stripped of eggs. The milt was removed using a 20 gauge needle and capillary tube inserted into the vent adjacent to the anal fin and stored in a 20ml universal tube at 4^{o}C. The viability of the milt was then assessed using a standard visual assessment of motility with the aid of a microscope (Bobe and Labbe, 2010). Based on motility and the volume of milt produced, milt form a single male from the control tank was selected to fertilise all eggs for the egg survival experiment.

5.3.5 Fertilisation of eggs and egg survival experiments

Approximately 200 eggs (estimated by weight) from each female were selected for further study. These were sub-divided into two replicates, and transported to S.C.E.N.E. (University of Glasgow Field station, Loch Lomond). Upon arrival the eggs were activated by fertilising them with milt from a single male, then placing them in individual incubation trays. Incubation trays were constructed from plastic mesh (5mm mesh diameter) wrapped round a solid square plastic frame (15cmx15cmx10cm). The trays were placed in a 1000L carbon fibre flume tank with 5 pipes located at one side of the system circulating water from Loch Lomond around the incubation area (15L min^{-1}). Waste water was drained by a standpipe screened by mesh located at the other end of the flume. The eggs were examined 3 times a week for mortalities and dead eggs, identified by their white/opaque appearance, were recorded and removed from the incubation system. The experiment was terminated when all eggs reached eyed stage of development. Water temperature during egg incubation ranged between 5^{o}C and 14^{o}C (mean= 8^{o}C).

5.3.6 UV-photolysis and determination of Se levels in eggs and livers

Eggs and livers from each female were freeze dried (Edwards Shelf Freeze Dryer) for 24 hr and homogenised using a mortar and pestle. Analysis of Se levels within samples was carried out at the Environmental Research Institute (ERI), University of the Highlands and Islands. Approximately 250mg of each sample was placed into 15ml tubes and pre-digested in 2ml of nitric acid overnight. Five blanks and five reference material samples were also made along with Se standards (20ppb, 40ppb, 80ppb, 110ppb and 320ppb) these too were pre-digested overnight in 2ml of nitric acid. Blanks contained 250µl mqH_2O and reference material samples contained 250mg of Dolt-4 (dogfish liver reference material for trace elements, Canadian Research Council). The accuracy of this method was assessed by determination of selenium content in the controlled reference material, Dolt-4. Samples were placed in a water bath for 2hrs at 100^oC and 250µl of H_2O_2 added every 5mins to prevent foaming during digestion. Samples were transferred into 20ml quartz tubes and placed in the UV-digester (Metrohm 750 UV digester). At 0, 30, 60 and 90min intervals, 200µl of H_2O_2 was added to the quartz tubes containing digested egg material. Clear, colour digests were obtained in all cases. Samples were transferred to 15ml tubes and diluted to 10ml with mqH_2O and selenium content analysed using a Varian 720-ES ICP-OES.

5.3.7 Chorion protein assay and SDS 1D-gel electrophoresis

The chorion was removed from ten un-activated eggs from each of the females selected for further study i.e. 10 fish on the control diet and 10 fish from each of 4 tanks of fish on the experimental diet. The extracted chorions from each female were subsequently pooled. They were then washed in pH 7.8, 0.2M Tris buffer for 30mins with three consecutive changes of buffer before being macerated in homogenisation buffer. The details of how the chorion was processed for SDS gel electrophoresis can be found in Chapter 2 (section 2.3.7) with the following amendments.

Due to the small amounts of protein present, the chorion samples in this experiment were subjected to an acetone precipitation. Approximately 1ml of acetone was added to an eppendorf containing 250µl of sample, and stored at -

20°C overnight. Samples were then centrifuged at 13,000rpm for 5 minutes at 4°C, which formed a pellet at the bottom of the eppendorf. Excess acetone was removed via pipetting and vortex drying at 40°C (Christ RVC 2-25. The pellet was then re-suspended in 100µl of chilled 0.01M phosphate buffered solution (PBS), and dissolved using pipette and vortex agitation before undergoing sonication (1 sec pulse, amplitude= 40) to ensure that it had completely dissolved.

The chorion protein concentration was measured using a modified version of the Lowry assay (Lowry *et al.*, 1951) after which, 5µg/ml from each sample was then prepared for protein profiling via SDS gel electrophoresis and run in duplicate for each individual female. Once electrophoresis was complete the gels were processed and stained using SilverQuest™ (Invitrogen). Finally, all clearly defined, reproducible protein bands were then scanned into the computer for image analysis using Scion imaging which calculates the pixel density of the selected band to provide a semi quantitative measurement of the protein concentration in each band. The pixel density of all gels were standardised using a reference gel which contained chorion samples from both control and experimental fed broodstock. In total 12 bands were analysed.

5.3.8 2D SDS gel electrophoresis

In order to determine if differences in the protein content were a result of changes in the concentration of proteins present or the presence of different proteins, 2D-gel electrophoresis was carried out. Samples processed for 1D-gel electrophoresis were pooled to create a single control and single experimental sample i.e. 10 protein samples from individuals fed the non-supplemented (control) diet and 40 protein samples from individuals fed the supplemented (experimental) diet.

Due to the high salt content, caused by the presence of 10% SDS from the homogenisation step, samples were acetone precipitated twice in an attempt to lower the salt content, and pellets containing protein re-suspended in 50µl 10mM Tris buffer. The pooled chorion protein concentrations were measured using the modified Lowry assay (Lowry *et al.*, 1951) and 100µg of protein placed in a 1.5ml eppendorf ready for electrophoresis.

Samples were suspended in 450µl of rehydration buffer (8M urea, 2M thiourea, 2% CHAPS, 0.02% bromophenol blue, 10mg of DTT, 5µl of IPG buffer), loaded into IPG strip holders together with dehydrated pH 4-7 non-linear IPG strips (Amersham Biosciences, UK) and covered with Drystrip cover fluid. Samples were focused using an IPG Phor II machine (Amersham Biosciences, UK). The strips were rehydrated at 20°C for 10-15hr. Iso-electric focusing (IEF) was then performed at 50mA/strip; applying 500V for 500V·h (Volt hours), 1000V·h, a linear voltage increase to 8000V (3975V·h) and 8000V for 70000V·h.

After IEF, IPG strips were equilibrated for 15mins in 10ml of equilibrium buffer (75mM Tris-HCl pH 8.8), 6M urea, 30% glycerol, 2% SDS, 0.01% bromophenol blue) containing 100mg of DTT, and then for a further 15min in 10ml of equilibrium buffer containing 250mg of iodoacetamide. Equilibrated strips were then placed on top of vertical slab gels and held in place by the addition of molten agarose. These gels were then loaded in a DALT 12 gel tank (Amersham Biosciences) filled with SDS electrophoresis buffer (25mM Tris-HCl, pH8.3, 192Mm glycine, 0.2% SDS) and electrophoresed at 15W per gel (at 25°C) for approximately 24hrs. Once electrophoresis was complete the gels were processed and stained using SilverQuest™ (Invitrogen).

5.3.9 Protein identification using mass spectrometry and bioinformatics

Spots from the 2-D gels were excised and the silver ions bound to the proteins removed using SilverQuest™ de-staining solution before being processed for in-gel trypsin digestion. The samples were then dehydrated in 100% methanol for 5mins and rehydrated in 30% methanol for 5mins at room temperature before being washed twice in ultrapure water for 5mins. The samples were subsequently washed a further three times with 100mM ammonium bicarbonate, containing 30% acetonitrile, for 10mins each. The samples were then dissected into smaller pieces and washed in ultrapure water before being dried in an ISS110 SpeedVac (Thermo Scientific) for 30 mins. The samples were then re-suspended in 50mM ammonium bicarbonate and 5ng/µl trypsin before being incubated overnight at 37°C. The resulting solution was then centrifuged for 1min before the supernatant was pipetted to a sterile eppendorf leaving the pellet. Finally the peptides and proteins within the pellet, were extracted with

10µl of 50% acetonitrile containing 0.1% trifluoroacetic acid at room temperature, combined with the supernatant from the previous step and concentrated to 4-5µl using a SpeedVac.

The differentially expressed proteins in each spot were identified by biological mass spectrometry at the Sir Henry Wellcome Functional Genomics Facility (University of Glasgow). Peptide tandem mass spectra were obtained by liquid chromatography-electrospray tandem mass spectrometry (LC-ES-MS/MS) as describe previously (Drummelsmith *et al.*, 2003; Besteiro *et al.*, 2004) using LCQ DecaXP (ThermoFinnigan) and ABI QStar (Applied Biosystems) quadropole ion trap instruments equipped with nanoelectrospray interfaces. The resulting peptide MS/MS spectra were used for interrogation of the NCBInr protein database using MASCOT software (http://www.matrixscience.com) against Actinopterygii (ray-finned fishes) data. The data searching criteria were set as follows: protein masses were unrestricted; peptide mass tolerance ± 0.3Da; max missed cleavage was 1.

5.3.10 Data analysis

All data presented as percentages were arcsine transformed before statistical analysis. Tank and dietary treatment effects for the fish were examined by nested one-way analysis of variance (ANOVA). Visual identification was used to determine differences in the chorion protein profile between treatment groups. Linear regression was used to assess the relationship between egg survival and egg Se concentrations and liver selenium concentrations. Minitab® 16 was used for data analysis.

5.4 Results

5.4.1 The effect of broodstock holding tank on Se levels and egg quality parameters

There was no significant effect of broodstock holding tank on egg survival, egg selenium concentration or liver Se concentration (Table 5-1). However, there was a significant tank effect in chorion protein concentrations for bands 1 ($F_{[3,49]}$= 16.60, r^2= 0.55, p< 0.001), 2 ($F_{[3,49]}$=5.96, r^2= 0.30, p= 0.002), 5 ($F_{[3,49]}$= 2.93, r^2= 0.22, p= 0.029) and 12 ($F_{[3,49]}$= 3.05, r^2= 0.19, p= 0.038) (Table 5-2).

5.4.2 The effect of dietary supplementation on egg survival

Eggs produced by salmon fed the supplemented diet had a significantly lower survival rate compared to fish fed the non-supplemented diet ($F_{[1,49]}$= 10.75, r^2= 0.25, p= 0.002).

5.4.3 Concentrations of Se in eggs and livers of Atlantic salmon fed supplemented versus non-supplemented diets.

Eggs form fish fed the supplemented diet contained significantly higher levels of Se compared to the eggs from salmon fed the non-supplemented diet ($F_{[1,49]}$= 6.65, r^2= 0.16, p= 0.013). There was no significant difference in liver Se concentrations between salmon fed supplemented and non-supplemented diets.

5.4.4 Relationship between egg survival and Se concentrations in the eggs and livers of Atlantic salmon

There was no significant linear relationship between egg Se levels and egg survival (r^2= 0.04, p=0.183) or liver Se levels and egg survival (r^2= 0.04, p= 0.164).

5.4.5 Dietary supplementation and chorion protein profiles and concentrations

Chorions from eggs produced by salmon fed the supplemented diet had significantly lower concentrations of proteins in bands 7 ($F_{[1,49]}$= 116.14, r^2= 0.72, p< 0.001), 10 ($F_{[1,49]}$= 8.64, r^2= 0.17, p= 0.005) and 11 ($F_{[1,49]}$= 5.59, r^2= 0.23, p= 0.022), but high concentrations of proteins in bands 1 ($F_{[1,49]}$= 4.40, r^2= 0.54, p= 0.042) and 8 ($F_{[1,49]}$= 14.10, r^2= 0.25, p< 0.001) compared to fish fed the non-supplemented diet.

There were no consistent differences in the number of protein bands observed in the chorions from eggs produced by salmon fed the non-supplemented or supplemented diets (irrespective of tank differences), during 1D-gel electrophoresis (Figure 5-1).

Table 5-1: Differences in egg survival, liver and egg Se concentrations between tanks containing Atlantic salmon fed the supplemented and non-supplemented diet.

Parameter	Tank 1 (Exp) (Mn ± Std.Dev)	Tank 2 (Exp) (Mn ± Std.Dev)	Tank 3 (Exp) (Mn ± Std.Dev)	Tank 4 (Exp) (Mn ± Std.Dev)	Tank 5 (Ctrl) (Mn ± Std.Dev)	Tank P-value	Treatment P-value
Egg survival (%)	59.31±12.56	60.04±8.88	53.57±8.18	51.63±17.13	69.36±7.30	0.274	**0.002**
Egg Se conc. (ppm)	1.64±0.27	1.54±0.17	1.54±0.17	1.62±0.18	1.40±0.17	0.563	**0.013**
Liver Se conc. (ppm)	33.81±7.87	34.52±15.77	39.23±10.25	32.48±5.64	27.84±6.91	0.452	0.051

Table 5-2: Differences in chorion protein concentrations between tanks containing Atlantic salmon fed the supplemented and non-supplemented diet.

Parameter	Tank 1 (Exp) (Mn ± Std.Dev)	Tank 2 (Exp) (Mn ± Std.Dev)	Tank 3 (Exp) (Mn ± Std.Dev)	Tank 4 (Exp) (Mn ±Std.Dev)	Tank 5 (Ctrl) (Mn ± Std.Dev)	Tank p-value	Treatment p-value
Protein band 1	7476±4572	8705±6105	83076±65682	4408±2698	3964 ±1638	**<0.001**	**0.042**
Protein band 2	5539±3421	7359±6151	11452±9053	90103±118381	3407 ±1489	**0.002**	0.187
Protein band 3	10110±6675	7315±5293	13288±11880	6535±4795	6031± 3609	0.197	0.197
Protein band 4	5864±2433	6241±2019	8242±4727	5052±3166	4955 ±2899	0.159	0.222
Protein band 5	11885±5887	11383±5975	13897±9286	5569±4298	6848 ±4399	**0.029**	0.089
Protein band 6	4828±1810	4677±1985	6011±4086	5342±4032	3756 ±2177	0.176	0.176
Protein band 7	1120±409	1222±467	1485±954	1126±598	10792± 5423	0.987	**<0.001**
Protein band 8	3166±1883	1892±1405	2244±1464	2043±1262	8075 ±9174	0.913	**<0.001**
Protein band 9	5654±2124	5576±3258	8765±5422	8949±5306	8003 ±6846	0.236	0.659
Protein band 10	4082±2326	1975±1654	3234±2602	3661±3484	10846± 15523	0.926	0.005
Protein band 11	13157±6879	3685±3279	7041±7577	6123±5822	13946± 12149	0.055	0.022
Protein band 12	8329±2661	2455±1892	6284±4626	6284±4626	8452± 5448	0.038	0.225

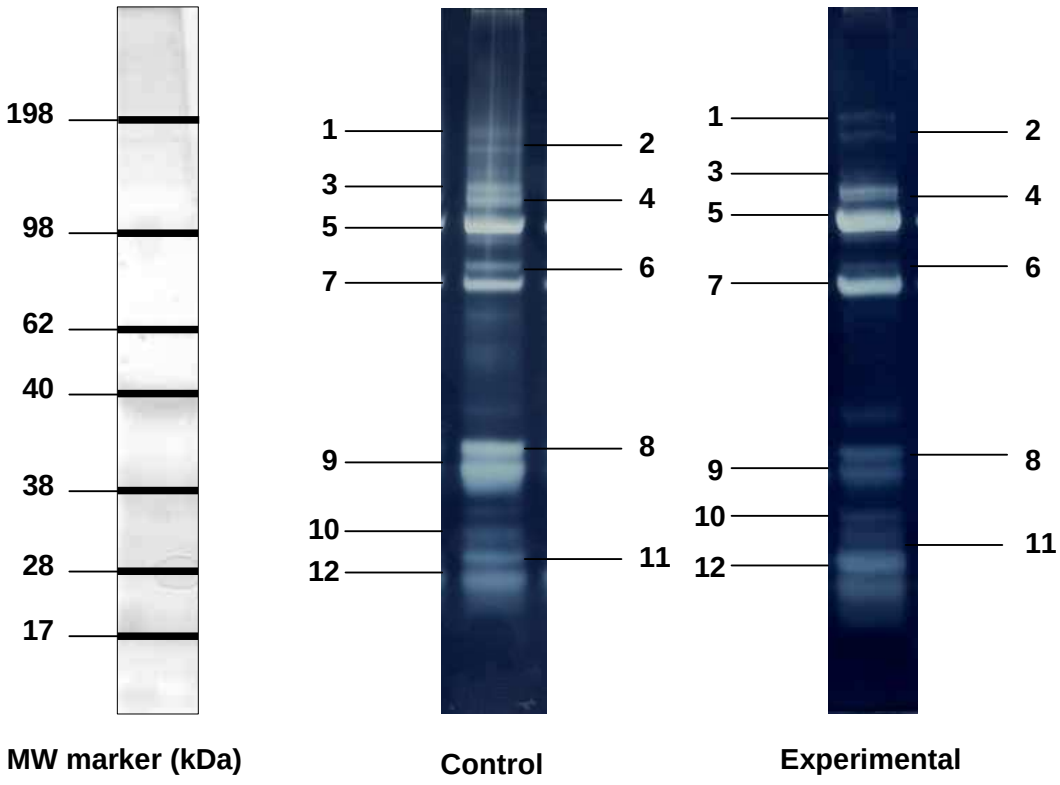

Figure 5-1: An example of the protein profile from the chorion of eggs from Atlantic salmon fed non-supplemented (control) and supplemented (experimental) diets. Numbers 1-12 indicate the bands selected for analysis.

The small number of protein spots recorded on the 2D-gel electrophoresis suggests that the procedure was not fully successful and that the results are inconclusive due to a lack of sensitivity in the procedure. Six spots representing proteins from the eggs of salmon fed Se-supplemented diets and 8 spots from salmon fed the non-supplemented diet were processed for identification, however there was no significant matches using the NCBInr protein database and MASCOT software (Figure 5-2 and Figure 5-3).

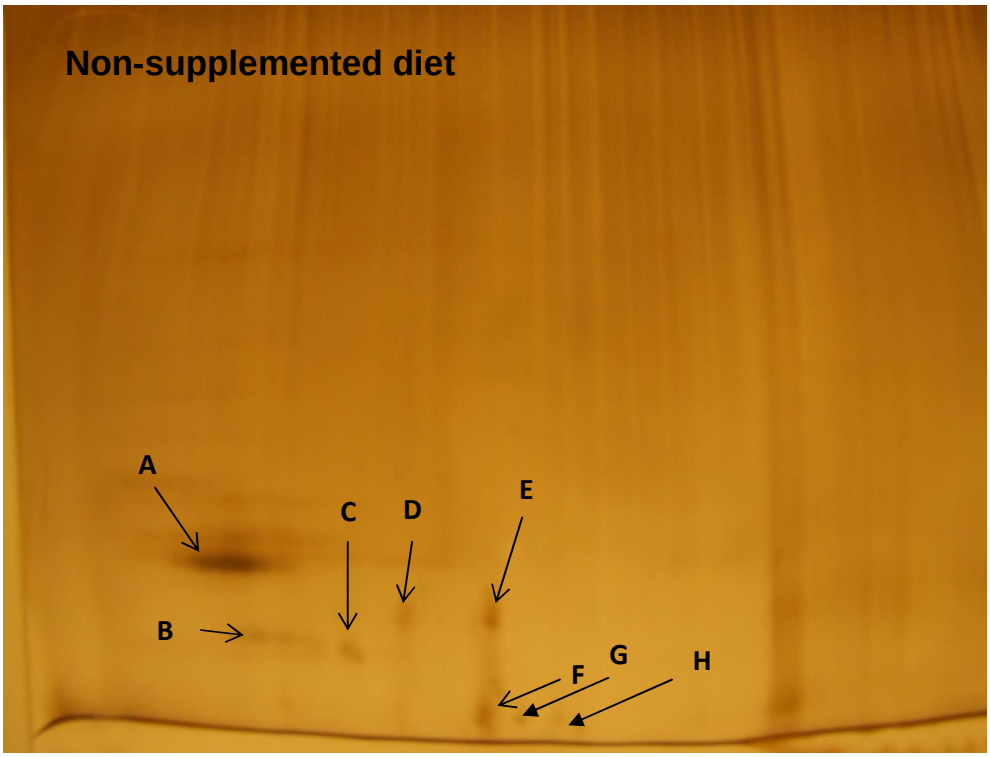

Figure 5-2: Chorion protein profile, using 2D SDS gel electrophoresis, of eggs produced by Atlantic salmon fed a non-supplemented diet. Letters A-H represent individual protein spots analysed using LC-ES-MS/MS.

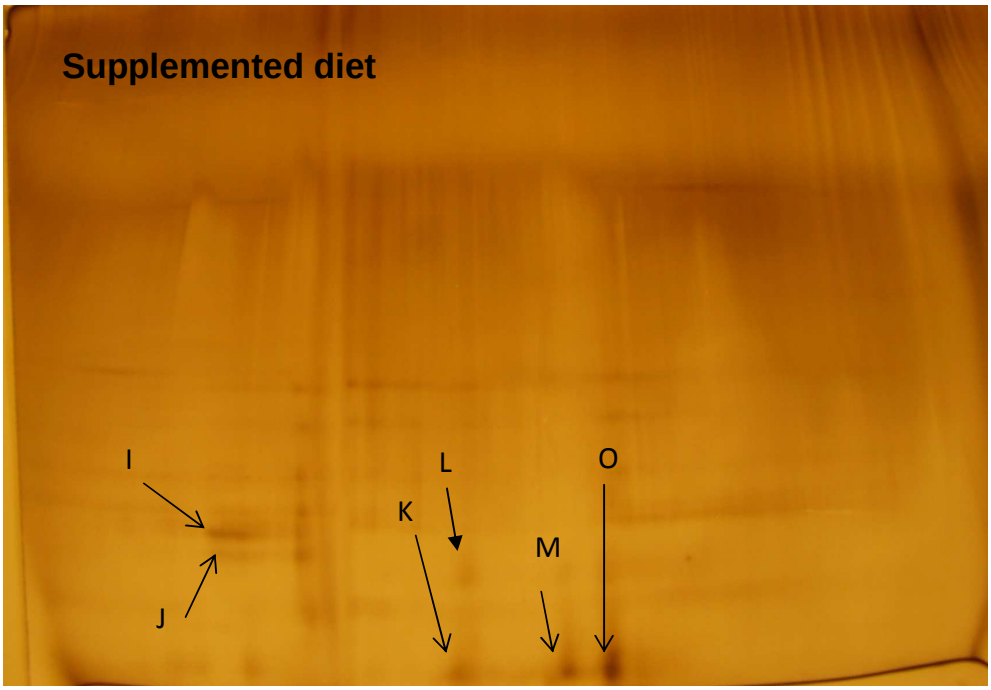

Figure 5-3: Chorion protein profile, using 2D SDS gel-electrophoresis, of eggs produced by Atlantic salmon fed a supplemented diet. Letters I-O represent individual protein spots analysed using LC-ES-MS/MS.

5.5 Discussion

The results indicate that Se from the Sel-plex supplemented diets, are stored in the liver of broodstock Atlantic salmon before being transferred to the eggs, pre-ovulation. While it is well known that the liver is the largest store of dietary Se in fish, this is the first study to indicate that dietary Se, in the form of Sel-plex, is transferred to the eggs of farmed Atlantic salmon held under commercial aquaculture conditions. The results also suggest that Skrettings 'Vitalis' supplemented diet altered the protein structure of the chorion and negatively impacted egg survival. Due to the lack of a linear relationship between egg/liver Se levels and egg survival it is possible that other constituents of the Vitalis diet were responsible for the significantly lower egg survival rates. Due to the unsuccessful attempt to identify the proteins present within the chorions of eggs produced by salmon fed supplemented and non-supplemented diets using 2D-gel electrophoresis, it was not possible to tell if differences in the proteins observed during 1D-gel electrophoresis were due to differences in the concentration of proteins or the presence of additional proteins in the chorions of eggs from fish fed supplemented or non-supplemented diets.

Information regarding the relationship between increased dietary levels of Se fed to fish and the increased Se content of eggs remains un-documented until now, however studies in poultry have shown similar results. For example, the inclusion of Sel-plex into the diet of breeder chickens (*Gallus gallus*) significantly increased the Se levels in the perivitelline membrane of their eggs (Surai *et al.*, 2004). Moreover, during a study examining the effect of dietary organic Se on the tissues of newly hatched quail, Karadas *et al.*, (2004) observed that Se concentrations within the quail eggshell also increased significantly when these animals were fed organic Se at a similar dietary level to those used during the current study.

Detailed knowledge concerning Se metabolism, specifically its uptake, storage and excretion in fish is fragmentary. Information concerning how dietary Se is metabolised by broodfish, and if it is transferred to their eggs has also, so far remained undocumented. The results observed during the current study suggest that dietary Se is incorporated into the eggs of Atlantic salmon and a possible mechanism for this transfer is suggested below. During the current study the

supplemented diet contained approximately 1.3mg/kg of Se, of which 0.5mg/kg was the organic Se compound Sel-plex. In dietary concentrations above 0.35mg/kg the liver contains the highest store of Se (Hilton *et al.*, 1982; Rider *et al.*, 2009). The liver is also the site for chorion and yolk protein production, such as vitellogenin and the zona radiata proteins, takes place. During normal metabolic processes organic Se compounds may bind to these proteins during the substitution of sulphur by Se in the amino acids cysteine and methionine to form selenocysteine (Se-Cys) and selenomethionine (Se-Met) (Arukwe and Goksoyr, 2003; Rider *et al.*, 2010). Selenium is also non-specifically incorporated into animal proteins via Se-Met, as methionine transfer RNA does not discriminate between Se-Met and its sulphur analogue (Schrauzer, 2003).

In support of this suggested mechanism of maternal Se transfer, Oppen-Berntsen *et al.*, (1992) found that primary liver cultures from female salmonids incorporated radioactive (^{35}S) methionine into four major proteins observed within their circulatory system prior to sexual maturation and that three of these proteins were specifically immuno-precipitated with antibodies to chorion proteins. After the yolk and chorion proteins have been manufactured and the Se molecules incorporated into their structures, they are transferred to the ovary via the circulatory system and incorporated into the developing egg (Arukwe and Goksoyr, 2003).

According to the manufactures website (www.skrettingnwe.com) the Vitalis diet is specifically designed for the demands of broodstock fish management and as such contains protein levels higher than normally found in diets tailored to increasing the growth rates of fish. Proteins act as a source of amino acids and as a reservoir of materials used during the many biosynthetic activities taking place during reproductive development and embryogenesis (Metcoff 1986). Previous studies have shown that type and quantity of protein can affect reproductive performance in a number of marine and freshwater fish species (Izquierdo *et al.*, 2001). For example, current aquaculture feeds contain proteins based on plant or animal (usually fishmeal) materials, plant based protein contains high levels of cellulose which has no nutritional value to carnivorous fish and is less digestible than fishmeal based diets (Francis *et al.*, 2001; McGoogan and Reigh, 1996; Sugiura *et al.*, 1998). Without an exact ingredient list for the Vitalis diet, which was unavailable, it is difficult to explain

the significantly lower survival rate of eggs from fish fed the supplemented diet, however it may be caused by an increase in the content of plant based protein which is not properly digested by the salmon and therefore not passed onto the eggs during development.

The results from the 1D-gel electrophoresis (1D-GE) suggest that the chorion from eggs produced by Atlantic salmon fed the non-supplemented diet contained either additional proteins or higher concentrations of proteins present compared to salmon fed the supplemented diet. In order to determine the basis of these differences samples were pooled for 2D-gel electrophoresis (2D-GE). Compared to 1D-GE, 2D-GE would be able to identify the presence or absence of specific proteins within the 12 bands produced during 1D-GE. If there was no difference in the protein profile after 2D-GE then we could conclude that the dietary treatment altered the concentration of proteins and not the number of proteins present. Attempts to investigate these results were unsuccessful as only 6-8 protein spots were found to appear after silver staining compared to the 12 bands which were produced during 1D-GE. A number of possible causes are suggested; however the most likely source of the difficulties is the presence of high salt concentrations (SDS and PBS solutions) within the samples during the initial processing procedures.

During 2D-GE the iso-electric focusing step is used to distinguish proteins according to their iso-electric point (pI) before they are subsequently separated by their molecular mass using SDS-gel electrophoresis. During the high voltage iso-electric focusing procedure, a small amount of residual salt ions is sufficient to retard the progression of the proteins across the 'strips', thereby worsening the protein focusing (Wu *et al.*, 2010). This was observed during the current study, as samples needed 48hrs instead of the usual 24hrs of focusing in the IPG Phor II machine, to obtain the required 8000V and 70,000V·h. In order to rectify this problem original chorion protein samples were acetone precipitated and the protein re-suspended in 50µl, 10mM Tris buffer, which contained less salt ions than the previously utilised 100µl 0.01M PBS. While the iso-electric focusing was subsequently completed within the recommended 24hrs the procedure was still unable to produce a clear protein profile of the chorion. It is thought that during the initial processing steps, the use of SDS contaminated the samples and may have introduced unsolicited and unpredictable artefacts, such as horizontal or

vertical streaks, protein migration drifts and gaps as well as various protein modifications in the corresponding 2D-gels (Berkelman 2008; Lee and Chang, 2009; Wu *et al.*, 2010). This theory is supported by the horizontal and vertical streaks viewed in the corresponding gel (Figure 5-2 and 5-3), while the inability of the NCBInr database and mascot software to indentify the protein spots that were observed are indicative of modifications to the structure of the protein caused by high salt concentrations within each sample.

In conclusion the role of organic Se supplementation in broodfish nutrition has so far, received very little attention, with the majority of studies focusing on the so called major dietary components i.e. lipid carbohydrate and protein. However the potential benefits shown in other domesticated breeding animals, such as poultry, and the recent studies showing the health benefits in fish suggest that in the right concentrations, Se may greatly benefit egg quality in farmed fish species. This study has provided the first evidence of vertical transmission of dietary Se from the female broodfish to their eggs, in farmed Atlantic salmon, and while a possible mechanism of this transfer has been suggested further research is required in order to accurately identify and assess the process.

The chorion is a highly conserved proteinaceous membrane within teleosts, vital in protecting the egg and developing embryo from mechanical and environmental stressor's (Arukwe and Goksoyr, 2003). The alterations to the protein profile observed during the current study may or may not be related to the significantly lower survival rate found in eggs produced by broodstock fed the supplemented diet. However without an improvement in the 2D SDS-gel electrophoretic procedure and the nutritional breakdown of Skretting Vitalis broodstock diet, further conclusions on the significance of these results is not possible.

6 The effect of egg selenium enrichment on resistance to *Saprolegnia*

6.1 Abstract

Chapter 5 showed that Atlantic salmon (*Salmo salar*) fed a supplemented diet produced eggs containing higher concentrations of Se. Eggs from 14 salmon fed the supplemented diet and 10 fed the non-supplemented diet were tested for their ability to resist saprolegniosis within a controlled environment, similar to incubation conditions used by the aquaculture industry. There was no significant difference in the presence/absence of infection, infection rate or survival between Se-enriched eggs produced by Atlantic salmon fed the supplemented diet and non Se-enriched eggs produced by Atlantic salmon fed the non-supplemented diet. Therefore, it was concluded that egg Se-enrichment, by supplementation of broodstock diet, does not alter the eggs resistance to saprolegnosis.

6.2 Introduction

Mycotic infections of farmed salmonid fish and eggs, primarily by the genus *Saprolegnia* represent a significant economic and welfare problem, which has been exacerbated lately due to restrictions on the use of the most effective fungicide available, malachite green (Pottinger and Day, 1999). Unfortunately replacements such as sodium chloride, potassium permanganate and formalin have not provided a successful alternative (Bly *et al.*, 1996).

Mass mortalities of fertilised eggs caused by *Saprolegnia* spp have been reported in all countries with significant salmonid fish production industries e.g. Chile, Japan, Norway and Scotland (Pottinger and Day, 1999). Members from the family Saprolegniaceae (often referred to as water moulds) share common taxonomic features with both fungi and algae (Stueland *et al.*, 2005). While all freshwater fish and their eggs are at risk of contracting saprolegnosis, salmonids are particularly vulnerable (Hughes 1994). Despite its impact on salmon aquaculture and significant research effort, the industry is still unable to successfully prevent infections on a regular basis (Thoen *et al.*, 2011).

The fish embryo developing within a healthy egg is protected by the chorion, a proteinaceous membrane which functions as a mechanical, chemical and pathogenic barrier (Brook *et al.*, 1997). The chorion contains its own 'immune system', which is capable of protecting the egg from various disease challenges. For example, Kudo and Teshima, (1991) found that chorion extracts contained molecules with anti-microbial activities, and that these molecules exerted an anti-pathogenic action on *Saprolegnia parasitica*. Such actions have been similarly recorded in the chorions obtained from the eggs of ayu (*Plecoglossus altivelus*), perch (*Perca fluviatilis*) and the common carp (*Cyprinus carpio*) (Kudo and Inoue, 1989; Kudo and Teshima, 1991; Paxton and Willoughby, 2000). However, when the chorion becomes compromised by mechanical damage and/or poor water quality, saprolegnosis often occurs. Its virulence (e.g. the growth of fungal hyphae) then enables it to quickly and effectively cross contaminate neighbouring healthy eggs as well (Stueland *et al.*, 2005; Theon *et al.*, 2011).

All components of the egg, such as the chorion and its anti-pathogenic properties are produced by the female, which obtains the necessary vitamins and minerals needed to form the egg from its diet (Brooks *et al.*, 1997). Previous studies have shown that supplementing broodstock diets with a number of components such as proteins, fats, amino acids and carbohydrates can have a significant impact on egg survival (Izquierdo *et al.*, 2001). Reviews of the subject point to trace minerals such as selenium (Se) as being an essential dietary component (Brooks *et al.*, 1997; Izquierdo *et al.*, 2001; Rider *et al.*, 2009). However, compared to other dietary components, such as those mention above information regarding the impacts of Se supplementation in fish are poorly understood (Beck 1996; 1997; Schrauzer 2000).

Research on several fish species, including salmonids, has established that disease resistance can be compromised by Se deficiencies particularly under high stress conditions, like those experienced by fish under cultured conditions (Rider *et al.*, 2009). Information regarding what effect Se supplementing broodstock diets has on the anti-pathogenic properties of eggs is not known, however, studies have shown that a variety of immune molecules are transferred from female to egg (Grindstaff *et al.*, 2003; Huttenhuis *et al.*, 2006; Swain and Nayak, 2009). Therefore the aim of this study was to examine the effect that

supplementing Atlantic salmon (*Salmo salar*) broodstock diet with the organic Se supplement Sel-plex® (Alltech Ltd) may have on the ability of Se-enriched eggs to resist saprolegniosis.

6.3 Materials and Methods

6.3.1 Samples and holding facilities of female broodstock

The location, holding facilities and assessment of reproductive status of female Atlantic salmon broodstock fed non-supplemented and supplemented diets were as described in Chapter 5. Ten eggs from each of the 10 females fed the non-supplemented diet were selected for use in this study. From the 40 females fed the supplemented diet, 10 eggs from each of the 14 females were selected at random and used during this study.

6.3.2 Saprolegnia challenge experiment

Twenty four 2L rectangular aquarium tanks were placed within a dark, constant temperature room set at 3^{o}C between January and February 2011. The tanks were left to equilibrate for 1 week before the start of the challenge experiment. At the bottom of each tank a 24 well plate was fixed with silicone and an air stone (3.5cm x 6cm) was used to increase the dissolved oxygen levels within each tank.

Twelve tanks were designated as experimental tanks (Exp) and twelve tanks as control tanks (Ctrl). One egg from each of the 10 females fed the non-supplemented diets (non Se-enriched eggs) and 14 females fed supplemented diets (Se-enriched eggs) was placed in each of the individual wells in a randomised pattern. All healthy eggs used during the study were at the eyed stage of development. Eggs infected with *Saprolegnia*, (identified by the cotton-like, white to greyish patches on the egg (Stueland *et al.*, 2005) were removed from the egg survival experiment (described in Chapter 5 Section 5.3.5) and held in a 4L rectangular tank at room temperature until needed for the challenge experiment, then placed into each individual well within the Exp tanks, so that each of the 24 wells contained a healthy egg and an infected egg (Figure 6-1).

The Ctrl tanks contained only healthy Atlantic salmon eggs (Figure 6-1). All eggs in both the Exp and Ctrl tanks where visually checked every alternate day for the presence of saprolegniosis. The infection rate (number of eggs infected over time) and survival rate of Se- enriched and non Se-enriched eggs held in experimental tanks was analysed at the end of the study. In this instance survival was defined as an egg which had hatched before the end of the study, which lasted 14 days.

6.3.3 Data analysis

For the purpose of data analysis all data presented as percentages were arcsine transformed before being analysed statistically. The effect of egg Se-enrichment on the presence of infection as well as infection and survival rates of eggs from individual fish was examined using one-way analysis of variance ANOVA by Minitab® 16.

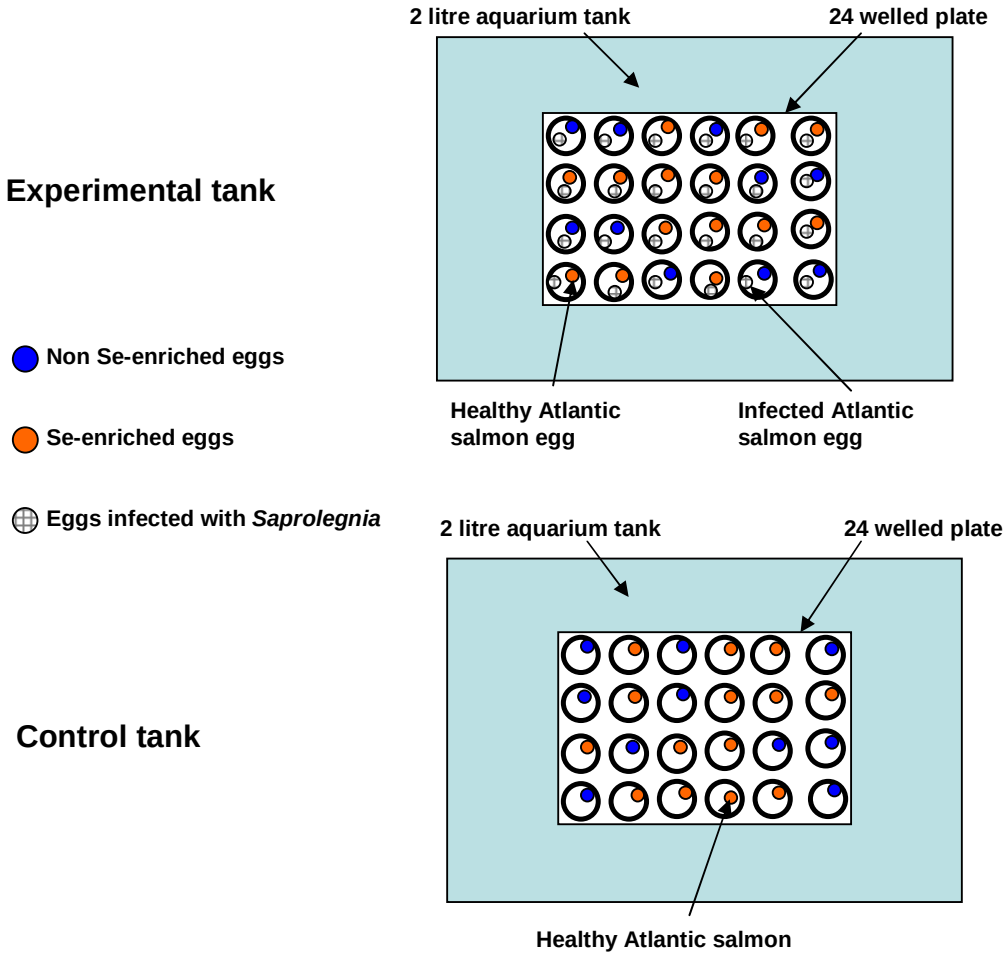

Figure 6-1: Atlantic salmon egg incubation system containing non Se-enriched eggs and Se-enriched eggs.

6.4 Results

6.4.1 The effect of incubation tank on the presence of infection on eggs

There was a significantly higher rate of infection in the Exp tanks (86.50% ± 6.96%) compared to Ctrl tanks (0.70%±3.43%) ($F_{[1,47]}$= 88350.7, r^2= 98.46, p< 0.01). As the number of eggs infected in the Exp tanks were significantly higher than the Ctrl tanks, no further analysis of eggs incubated within the Ctrl tanks was required. This also confirmed the effectiveness of the challenge experiment.

6.4.2 The effect of egg Se-enrichment on, presence of infection, infection rates and survival rates of eggs

Within Exp tanks there was no significant difference in the presence/absence of infection, infection rates or survival between Se-enriched and non Se- enriched eggs from Atlantic salmon (Figure 6-2; Table 6-1). After 22 days almost all eggs in both enrichment groups were infected with *Saprolegnia*, however some eggs survived despite being infected (Table 6-1; Figure 6-2).

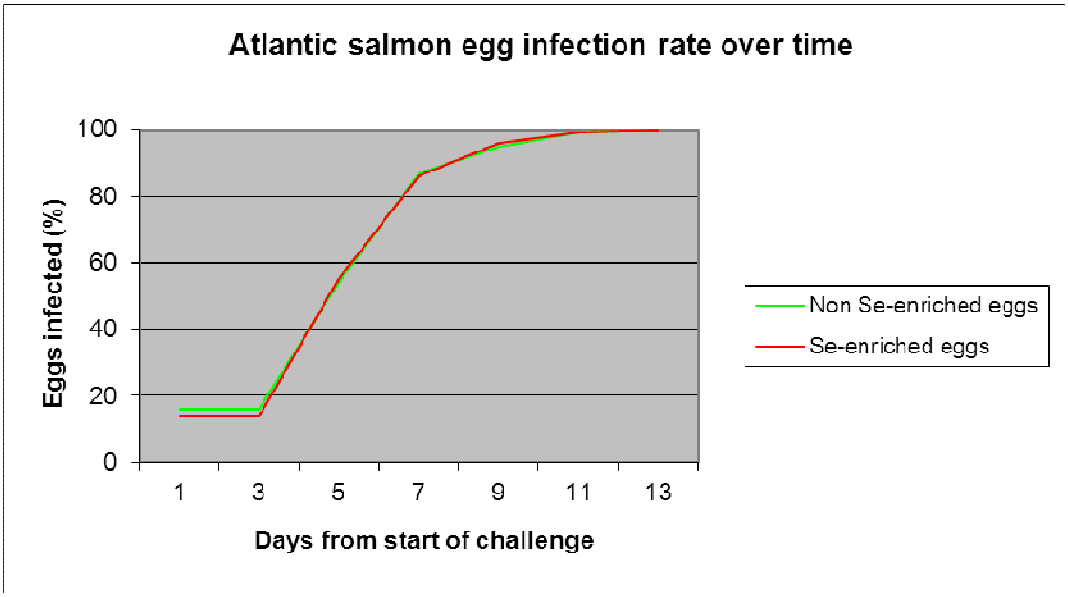

Figure 6-2: The rate of infection of non Se-enriched and Se-enriched Atlantic salmon eggs.

Table 6-1: Egg survival and infection percentages from non Se-enriched and Se-enriched Atlantic salmon eggs.

Parameter	Non Se-enriched eggs (Mn ± Std dev)	Se-enriched eggs (Mn ± Std dev)	P-value
Egg infection (%)	86.64 ± 7.08	86.41 ± 7.15	0.936
Egg survival (%)	59.91 ± 7.68	62.77 ± 5.51	0.298

6.5 Discussion

The results suggest that the increased concentration of Se in the eggs of Atlantic salmon fed the supplemented diet had no effect on 1) the ability of their egg's to resist saprolegniosis 2) the speed at which *Saprolegnia* spp infected eggs and 3) the survival rates of eggs challenged with *Saprolegnia*. This is the first study investigating the effect Se has on an egg's ability to resist a *Saprolegnia* infection.

Previous studies have shown that dietary Se increases the immune functions of fish and that the chorion has anti-pathogenic properties (Gatlin *et al.*, 1986; Kudo 2000; Kudo and Inoue, 1986; Sohn *et al.*, 2000; Thorarinsson *et al.*, 1994; Wise *et al.*, 1993). The working hypothesis tested here, that vertical transfer of immunity related molecules (female to egg) would enable Se-enriched eggs from supplemented broodstock to resist a *Saprolegnia* infection, was not supported by this study. A possible explanation is the significant difference in pathogenicity between different species and strains of *Saprolegnia* (Stueland *et al.*, 2005). The Se-plex supplemented diet fed to Atlantic salmon may have increased the Se-enriched eggs resistance to less virulent species, for instance *Saprolegnia diclina*, but was unable to prevent infection from highly pathogenic strains, such as *Saprolegnia parasitica*. Thoen *et al.*, (2011) found that strains which were normally pathogenic to Atlantic salmon parr were not particularly pathogenic towards eggs, and that strains that gave the highest infection rates to eggs were considered saprotrophs. Identification to species level of *Saprolegnia* strains is difficult and requires identifying the presence of sexual structures microscopically (Stueland *et al.*, 2005). The criteria used during this experiment to identify the presence of *Saprolegnia* was not detailed enough for species level identification and such a specific examination of sexual structures was out with the remit of the study.

Alternatively, the lack of disease resistance shown by Se-enriched eggs from supplemented fish may suggest that the effect of this dietary supplement affected the egg contents and not the chorion. Once eggs are ovulated they take up very little, if any macromolecules, all contents of the egg are incorporated during maturation when they are still attached to the ovary (Brooks *et al.*, 1997). Many of these macromolecules constitute the machinery for post-fertilisation cell division and protein synthesis, i.e. DNA and RNA polymerases, histone proteins, translation and transcription factors etc (Brooks *et al.*, 1997). Selenoproteins identified in fish include glutathione peroxidase (GSH-Px), catalase, superoxide dismutase and thioredoxin reductase (TR), all of which are important molecules involved in intracellular antioxidant defence and provide reducing equivalents for many redox-dependent systems, such as DNA synthesis (Rider *et al.*, 2009). Instead of these molecules altering the disease resistance potential of the chorion they may be transferred from the female to the egg and sequestered into the yolk where they would benefit the embryo during the later stages of embryogenesis (hatching to first feeding).

There was no evidence to suggest that yolk Se in the form of molecules such as GSH-Px had any anti-pathogenic effect on the egg from fertilisation to hatching, but any potential benefits beyond these stages i.e. from the yolk sac stage and onto first feeding cannot be proven. Like birds and reptiles, many of the maternal immune factors are transferred through the yolk in fishes (Swain and Nayak, 2009). Immune molecules within the yolk such as IgM and complement components are vitally important in protecting newly hatched juveniles from infectious agents (Swain and Nayak, 2009). The availability of maternally derived Se in the yolk of juvenile fish may also be vitally important, especially as their lymphoid organs and ability to develop certain antibodies are still extremely under-developed weeks after hatching (Koumans-van Diepan *et al.* 1994; Lovoll *et al.*, 2006; Magnadottir *et al.*, 2004; Zapata *et al.*, 2006).

In conclusion, saprolegnosis continues to cost the aquaculture with losses of both fish and eggs recorded each year (Pottinger and Day, 1999). The success of current treatments is considered variable at best, therefore there is also continued research costs incurred while searching for new more effective treatments (Pottinger and Day, 1999). While the results from this study suggest that Se-enriched eggs are unable to prevent saprolegniosis, the species of the

infecting organism could not be identified. Also information regarding how dietary Se is metabolised, transferred to the ovary and stored within the egg is still lacking. More information is required concerning these subjects in order to accurately assess the potential of broodstock dietary supplementation to increase the eggs resistance to saprolegniosis.

7 Adhesive mechanisms in European whitefish (*Coregonus lavaretus*) eggs aid survival in high energy spawning grounds

7.1 Abstract

European whitefish (*Coregonus lavaretus*) populations in the British Isles are critically endangered and wave induced mortality during the egg stage of the lifecycle is thought to influence population numbers. However, adhesive mechanisms on the surface of the chorion may allow European whitefish eggs to remain on optimal spawning grounds despite the highly energetic environment. Our experiments show that whitefish eggs remain non-adhesive in a solution chemically similar to ovarian fluid, but become adhesive seconds after contact with water. Examination of the ultrastructure of the chorion showed that the morphology altered significantly after contact with water with nodule-like protuberances attached to connective filaments on the surface present in water hardened but not non-water hardened eggs. Biochemical analysis showed the presence of Chain A, RNase ZF-3e proteins in the chorion of water hardened but not non water hardened eggs. Histochemical staining of the chorion of *C. lavaretus* showed that the externa, but not the interna stained positively for the presence of glycoproteins. The potential benefit of European whitefish producing adhesive eggs is that they remain on the optimal spawning habitat, where factors such as predation, desiccation and hypoxia are minimised

7.2 Introduction

Due to declining numbers and threats to extant populations, the European whitefish (*Coregonus lavaretus*) has high conservation status in the UK, it is a UK Biodiversity Action Plan (UKBAP) species and listed on schedule 5 of the UK Wildlife and countryside Act 1982. Brown *et al.*, (1991) showed that 99.95% of whitefish eggs from a population in Loch Lomond do not survive on the exposed gravel banks or shorelines on which they are laid. This and other studies on UK European whitefish populations suggest that egg survival is a significant bottleneck to recruitment for this species (Brown and Scott, 1994; Etheridge *et al.*, 2011).

In many fish species egg survival is enhanced by either adult guarding behaviour, as found in bullheads (*Cottus gobio*), or the construction of nests as found in many other salmonid species (Youngson *et al.*, 2011). However, European whitefish neither guard their eggs nor build nests; instead eggs are only protected by the substrate upon which they fall after spawning. Thus, the main cause of European whitefish egg mortality includes eggs being swept off spawning grounds into unsuitable habitat and wave damage (Slack *et al.*, 1957; Ventlingschwank and Livingstone, 1994). Depositing eggs in areas with high wave energy produces low egg survival rates; therefore, this is likely to result in the rapid selection for egg characteristics which improve survival by enabling the egg to maintain its original position (Crossland and Shine, 2011; Martin and Swiderski, 2001).

The chorion is a proteinaceous membrane which surrounds the eggs of teleosts, protecting them from mechanical damage, desiccation, rapid chemical changes in the environment and pathogens, whilst simultaneously facilitating gas exchange for the developing embryo (Brooks *et al.*, 1997; Esmaeili and Johal, 2005; Kudo 1992; Yamagami et al., 1992). In general, the structure of the chorion includes a thick inner layer (the zona radiate interna (ZRI)), and a thin outer layer (the zona radiata externa (ZRE)) (Huysentruyt and Adrianens, 2005). Eggs from many species of fish share this structure, however, the morphology of the chorion exhibits modifications depending on the species and the environment (Arukwe and Goksoyr, 2003; Riehl and Patzner, 1998). One such modification to the chorion is the presence of adhesive mechanisms.

Knowledge of European whitefish egg structure is limited, however anecdotal evidence suggests that eggs may possess adhesive properties. The mechanism of egg adhesion, biochemical composition and ultrastructure of the chorion of European whitefish has not been fully investigated. The study reported here examined adhesion in European whitefish eggs and the potential physical and biochemical mechanisms which produce the adhesive activity. This information provides a better understanding of fish egg adhesion in general but specifically provides an insight into the mechanisms for avoiding wave induced mortality for this high conservation value species.

7.3 Materials and Methods

7.3.1 Egg collection

Ovulating female European whitefish were collected by Nordic pattern gill nets comprising 12 panels ranging from 5-55mm knot to knot mesh on presumed spawning grounds in Loch Eck, Scotland (Adams, unpublished; Brown and Scott, 1994; Etheridge *et al.*, 2010). To check for reproductive maturation, individual fish were removed from the nets and placed into 15L tanks containing the anaesthetic benzocaine (Sigma life Sciences) and checked for signs of abdominal distension and egg release. Ovulating fish were placed in a 15L bucket containing a lethal dose of anaesthetic, killed by a sharp blow to the head, blotted dry and their eggs stripped by abdominal manipulation into clean dry plastic tubs. Non-ovulating fish were placed in a portable floating cage system (2m x 2m x 1.5m depth) in Loch Eck and allowed to recover from anaesthesia before being released. Plastic tubs, each containing eggs from a single female were loaded into cool boxes and transported to the Scottish Centre for Ecology and the Natural Environment (S.C.E.N.E.), University of Glasgow.

7.3.2 Egg adhesion experiment

To investigate the presence of adhesive mechanisms on eggs of European whitefish, and to examine when adhesion initiates, two batches of twenty, un-fertilised and non-water hardened eggs from each female were placed into 2 glass petri dishes. One petri dish was filled with 10mls of water (from Loch Lomond) and allowed to water harden, while the other dish was filled with 10mls of artificial ovarian fluid (300mmol/l NaCl) (Mansour *et al.*, 2009a). An orbital shaker was used to simulate wave action by producing horizontal movement to agitate the eggs and liquids within both petri dishes. Horizontal movement was recorded as rotations per minute (rpm). Both petri dishes were placed on an orbital shaker at a speed of 70rpm and the number of eggs adhering to the substrate or other eggs was recorded after 30s 60s, 300s and 600s. Orbital shaker rotation was then increased to 100rpm and the number of eggs adhering recorded after 30s.

7.3.3 Preparation of chorion for histochemical examination

Twenty eggs from each of the 10 female Loch Eck European whitefish were collected in order to examine the microscopic anatomy of the chorion and to identify any chemical adhesive mechanisms. Ten non-water hardened eggs and ten water hardened eggs from each female were punctured with a sterilised needle. The yolk was then extruded by manipulation of the chorion. The chorions were placed into an eppendorf containing buffered neutral formalin (BNF), and after trimming were dehydrated, cleared and impregnated with paraffin wax. The paraffin-embedded sections were cut at 5µm thick sections using a microtome (Leica RM2125RT) and mounted onto glass slides. Alcian blue and periodic acid schiff (PAS) stains for glycoproteins and acid-mucopolysaccharides respectively were selected based on their presence on the chorions of adhesive eggs from other species (Chang and Huang, 2002; Galliano *et al.*, 2003; Mansour *et al.*, 2009a; Mansour *et al.*, 2009b; Riehl and Patzner, 1998). Chorions were subsequently examined using a light microscope (Leica DC480) at x40 and x60 magnification

7.3.4 Preparation for ultrastructure investigations using Scanning Electron Microscopy (S.E.M.)

To study the ultrastructure of European whitefish eggs and investigate the presence of mechanical adhesive mechanisms, five non-water hardened and five water hardened eggs from each female were washed in 0.1M cacodylate buffer then fixed in Karnovsky's solution for 24hrs. Eggs were dehydrated in 70% acetone for 4 hrs, 90% for 2 hours and absolute acetone for 12hrs. The eggs were then removed from acetone, critical point dried (Emitech K850), mounted onto aluminium stubs using double sided sticky carbon tabs and coated in gold palladium in an atmosphere of argon (Leica EM SCD005 sputter coater) for 4 mins. Subsequent analysis of egg ultrastructure was carried out using Philips XL30 ESEM and Cambridge Stereoscan 250 Mk3 microscopes (operating voltage = 10-20kv, working distance =7-10mm)

7.3.5 Chorion protein analysis using SDS gel electrophoresis

The molecular composition of the chorion of European whitefish eggs was examined using sodium dodecyl sulphate (SDS) gel electrophoresis on 10 water

hardened and 10 non-water hardened chorions from each female. Eggs were punctured with sterilised forceps, and the yolk removed via manipulation of the chorion. Chorions were then washed in pH 7.8, 0.2M Tris buffer for 30mins with three consecutive changes of buffer, before being macerated in 1ml of homogenisation buffer (1M Tris pH 7.4; 10% SDS; ultra-pure water; protease inhibitor cocktail (Sigma Life Sciences)) and transferred to a 1.5ml eppendorf to be stored at -80^{o}C until required.

Chorion protein concentrations were measured using a modified version of the Lowry assay (Lowry *et al.*, 1951). The chorion sample from each female was prepared using 1ml of BCA reagents (Thermo Scientific), which determines the protein concentration via a colorimetric reaction. A bovine serum albumin (BSA) kit (Thermo Scientific) provided a known protein concentration which was used to estimate sample concentrations using a standard curve. Each chorion sample was incubated at 37^{o}C for 30 mins in a water bath then transferred to cuvettes and light absorbance read at 562nm using a CE-1011 spectrophotometer (Cecil Instruments Ltd).

For SDS gel electrophoresis, 2µg/ml of protein from each chorion sample was transferred to 1.5ml eppendorf with 8µm of 3x sample buffer solution (3x sample buffer and 1M dithiotreitol (DTT)) and ultra-pure water to make a final volume of 25µl. The chorion sample was then heated at 90^{o}C for 4 mins (Tempette Junior TE-8J water bath). Pre-cast NuPage 4-12% bis-tris, 10 well, 1.0mm thick gels (Invitrogen) were loaded into a Novex mini-cell (Invitrogen) with 20% NuPage MES SDS running buffer (Invitrogen). Chorion samples from each female were then pipetted into individual wells along with SeeBlue pre-stained standard (Invitrogen). The SDS gel electrophoresis mini-cell was run at 90v for 2 hours, rinsed in ultra-pure water for 1 minute and placed on a Vibrax-VXR agitator (IKA Ltd) and immersed in 100ml of fixative solution (40% ethanol, 10% acetic acid and 50% ultrapure water) overnight. Gels were subsequently stained with SilverQuest™ (Invitrogen), scanned and documented.

7.3.6 In gel digestion

In order to harvest proteins from the SDS-gel electrophoresis, a band from the gel was excised and the silver ions bound to the proteins removed using

SilverQuest™ de-staining solution before being processed for in-gel trypsin digestion. The gel band was dehydrated in 100% methanol for 5 mins and rehydrated in 30% methanol for 5 mins at room temperature before being washed twice in ultrapure water for 5 mins. The gel band containing proteins was washed a further three times with 100mM ammonium bicarbonate, containing 30% acetonitrile, for 10 mins each. The gel band was then dissected into smaller pieces and washed in ultrapure water before being dried in an ISS110 SpeedVac (Thermo Scientific) for 30 mins. The dissected pieces were then re-suspended in 50mM ammonium bicarbonate and 5ng/µl trypsin before being incubated overnight at 37^{o}C. The resulting solution was then centrifuged for 1 min before the supernatant was pipetted to a sterile eppendorf leaving the pellet. Finally the peptides and proteins within the pellet, were extracted with 10µl of 50% acetonitrile containing 0.1% trifluoroacetic acid at room temperature, combined with the supernatant from the previous step and concentrated to 4-5µl using a SpeedVac.

7.3.7 Protein identification using mass spectrometry and bioinformatics

The differentially expressed protein was identified by biological mass spectrometry at the Sir Henry Wellcome Functional Genomics Facility (University of Glasgow). Peptide tandem mass spectra were obtained by liquid chromatography-electrospray tandem mass spectrometry (LC-ES-MS/MS) as describe previously (Drummelsmith *et al.*, 2003; Besteiro *et al.*, 2004) using LCQ DecaXP (ThermoFinnigan) and ABI QStar (Applied Biosystems) quadropole ion trap instruments equipped with nanoelectrospray interfaces. The resulting peptide MS/MS spectra were used for interrogation of the NCBInr protein database using MASCOT software (http://www.matrixscience.com) against Actinopterygii (ray-finned fishes) data. The data searching criteria were set as follows: protein masses were unrestricted; peptide mass tolerance ± 0.3Da; max missed cleavage was one.

7.4 Results

7.4.1 European whitefish egg adhesion

Eggs adhered with each other and with the wall of the Petri-dish after mixing with water. The majority of eggs, 57%±23% (mean ± standard deviation) adhered after 30 seconds (s); the maximum number of eggs adhering to other eggs and/or the petri dish was achieved between 60s and 300s (65% ± 23%). The number of eggs adhering did not increase or decrease thereafter (Figure 7-1). Adhesion was disrupted in all sticky eggs when simulated wave action was increased from 70rpm to 100rpm. No non-water hardened eggs immersed in 300mmol/L of NaCl adhered to either each other or the glass petri dish.

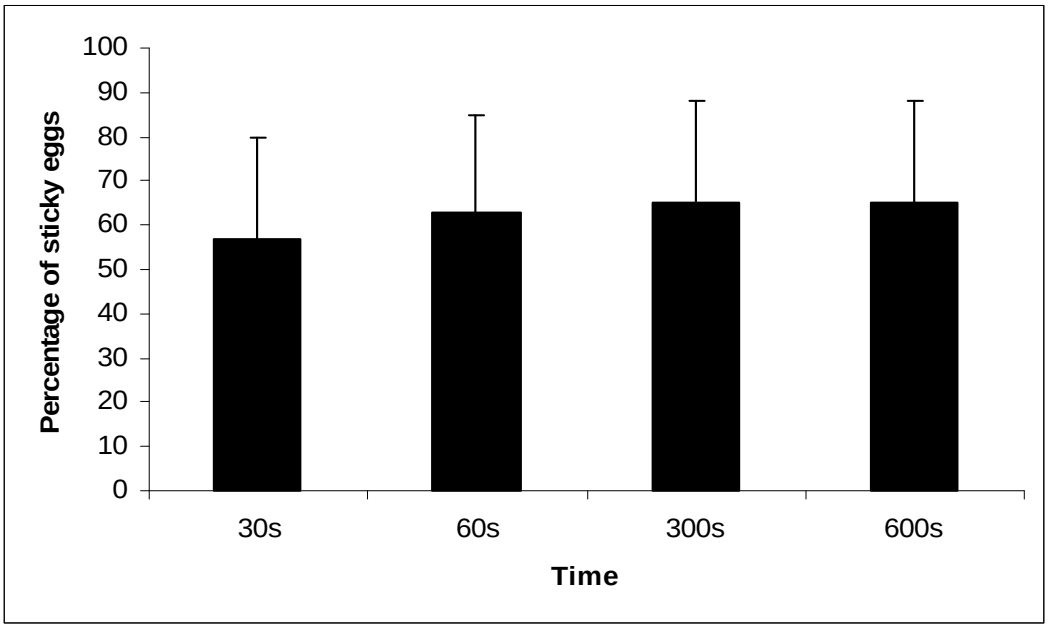

Figure 7-1: Effect of time on adhesion in water hardened *C. lavaretus* eggs (mean ± standard deviation).

7.4.2 European whitefish chorion ultra-structure

Scanning electron micrographs revealed significant differences between pre and post water hardened eggs. Nodule-like structures (1-3µm length) were observed protruding from the uneven surface on all non-water hardened eggs (Figure 7-2). Nodule-like protuberances were non-symmetrical in shape and irregularly spaced around the surface (Figure 7-2). The bases of these structures merged with the surface of the chorion (Figure 7-2). On water hardened eggs, nodule-like protuberances were attached to the surface of the chorion by filament-like

connections (1-3µm in length) (Figure 7-3). Underneath the nodule-like protuberances on water hardened eggs, a lattice work of connecting filaments could be seen (Figure 7-3). The uneven surface viewed on non-water hardened eggs was replaced with ridges running between protuberances on the surface of water hardened eggs (Figure 7-3). A granulated substance on the surface of chorion was regularly seen on micrographs of water hardened eggs and less regularly viewed on non-water hardened eggs (Figure 7-4). The granulated material did not cover the entire egg, but were observed in batches either on-top of nodule-like protuberances or in interstitial spaces (Figure 7-4).

7.4.3 European whitefish chorion histochemistry

In both non-water hardened and water hardened eggs the ZRE, but not the ZRI, stained positively with Alcian blue indicating the presence of glycoprotein's (Figure 7-5). Both the ZRE and ZRI stained positively with PAS indicating the presence of acid mucopolysaccharides; however the intensity of the stain on the ZRE was stronger (Figure 7-5).

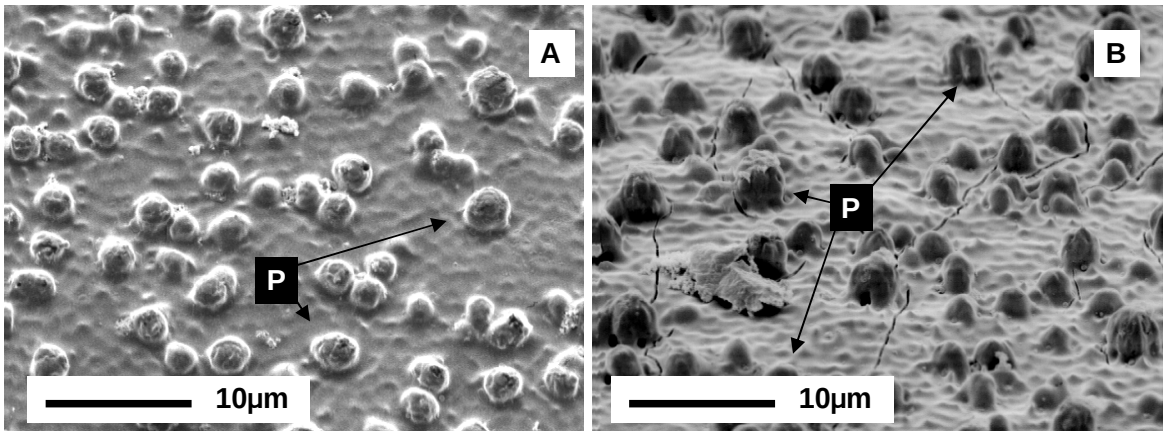

Figure 7-2: S.E.M. of the surface of non-water hardened *C. lavaretus* chorions. A) Non symmetrical nodule-like protuberances (P) on the surface of an egg (x2000). B) Lateral view of protuberances (1-2µm in length).

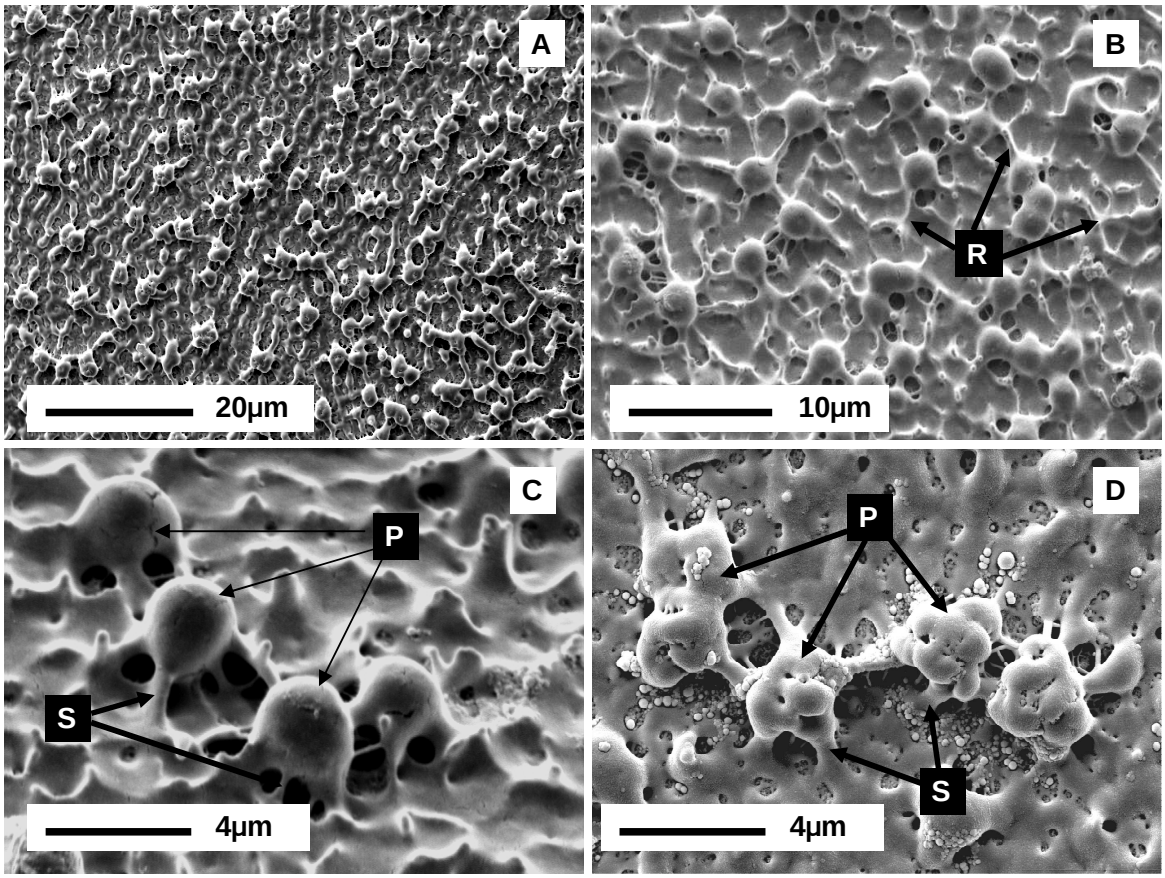

Figure 7-3: S.E.M. of the surface of water hardened *C. lavaretus* chorions. A+B) Ridges (R) run along the surface of the chorion between protuberances (x1000-2000). C+D) Protuberances have been raised from the surface of the chorion on filament-like connections (F) (x5000). Area underneath protuberances is exposed and lattice work of connecting filaments can be seen.

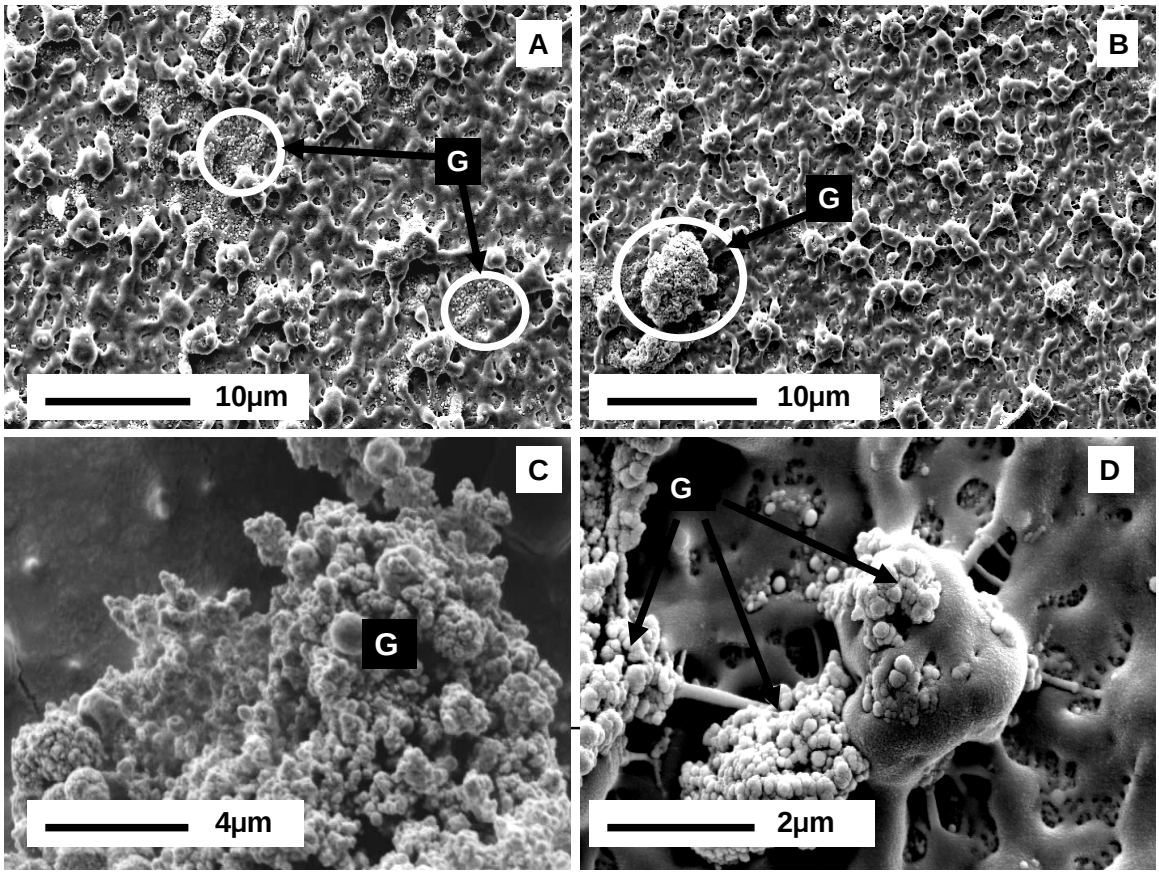

Figure 7-4: Granulated layers on *C. lavaretus* chorions, viewed using S.E.M. A) Granules (G) located in interstitial spaces between protuberances (x2000). B) Granules located on top of protuberance (x2000). C) Mat of granules on the surface of a chorion (x5000). D) Protuberance with granules (x5000).

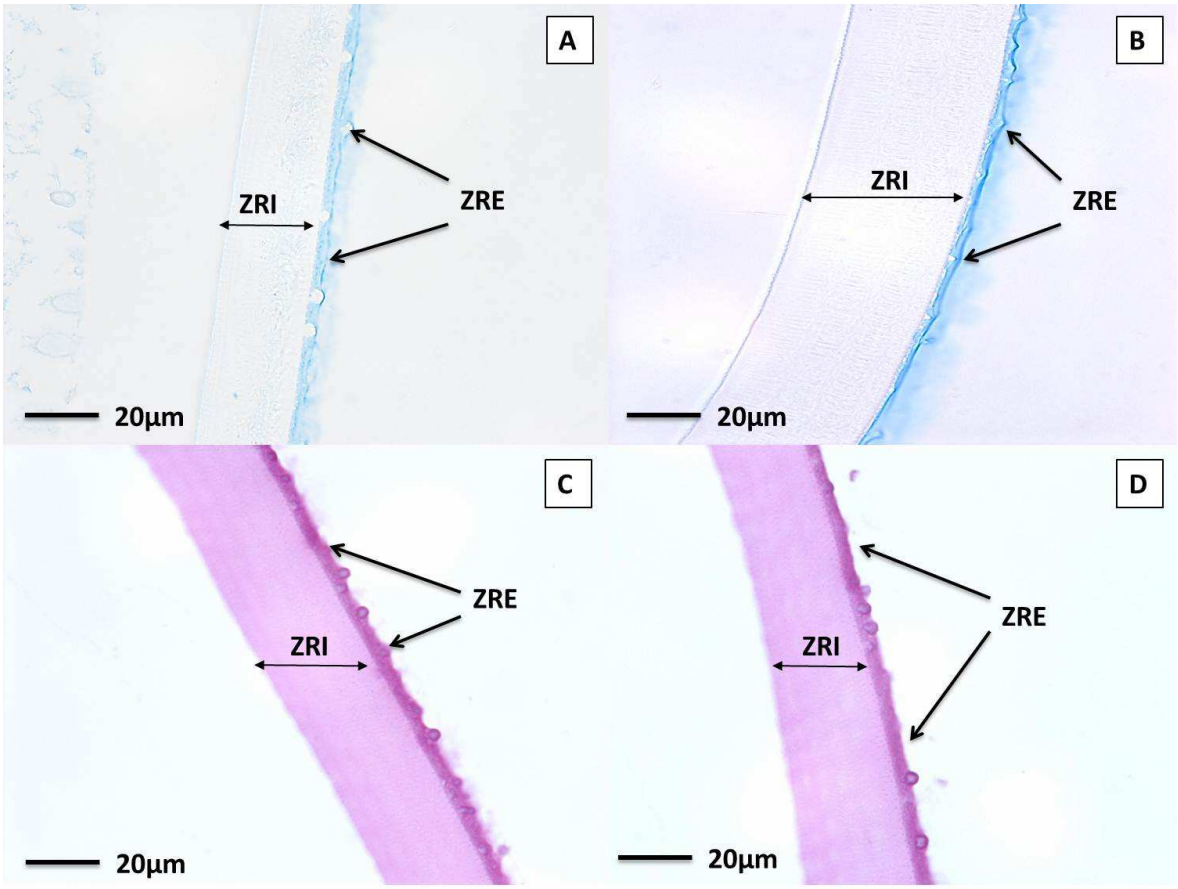

Figure 7-5: Histological sections from C. lavaretus chorions. A) Water hardened chorion stained with Alcian blue and showing the zona radiata interna (ZRI) and zona radiata externa (ZRE). B) Non-water hardened chorion stained Alcian blue. C) Water hardened chorion stained with PAS. D) Non-water hardened chorion stained with PAS.

7.4.4 Chorion protein profile of European whitefish eggs

The protein profiles of chorions from non-water hardened and water hardened whitefish eggs were compared visually. The qualitative protein composition of chorions from non-adhesive, non-water hardened eggs was similar to water hardened adhesive eggs, however, a protein band was observed at approximately 30kDa on water-hardened eggs but not non-water hardened eggs (Figure 7-6). The proteins found within the 30KDa band were identified from the NCBInr database (Table 7-1). Chain A, RNase ZF-3e had the highest number of peptides matched followed by translation initiation factor IF-2 and vitellogenin (Table 7-1).

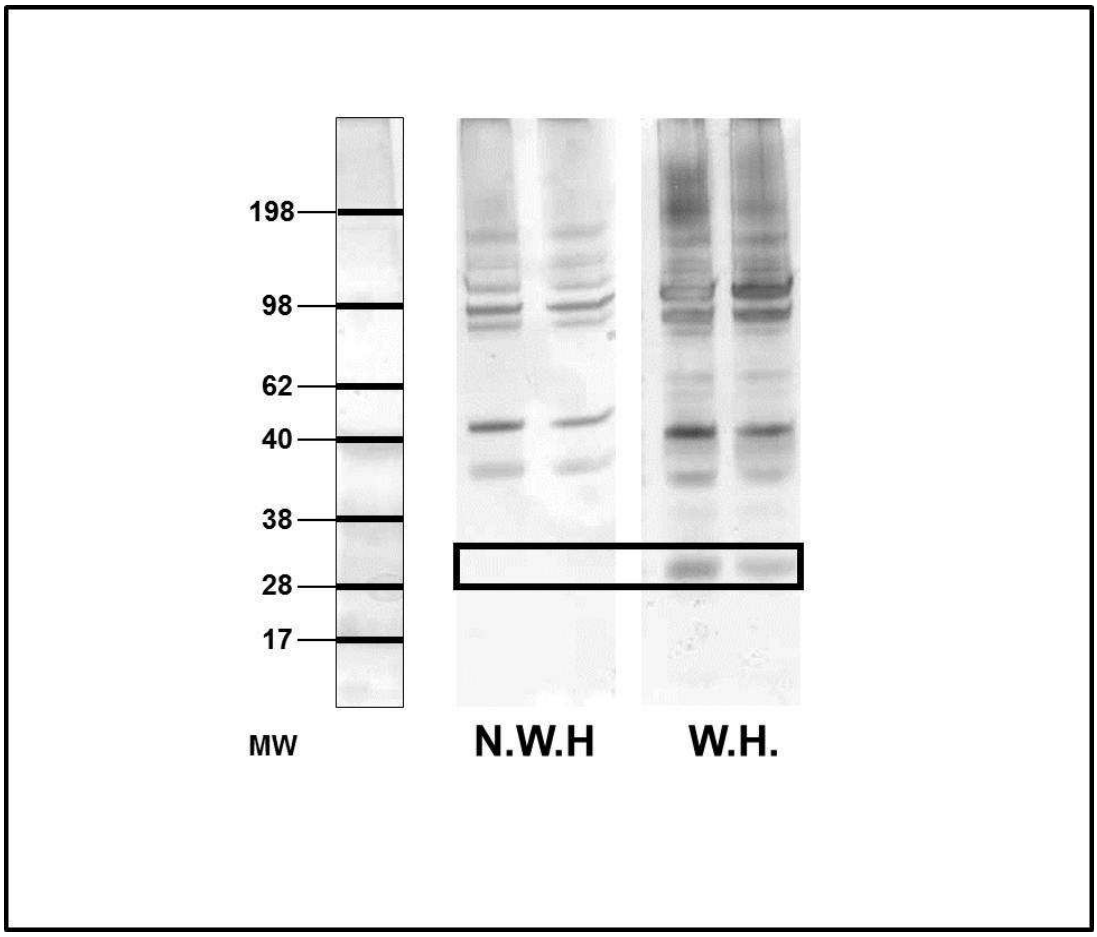

Figure 7-6: Protein profiles of the chorions from European whitefish eggs. Molecular weights (MW) are presented in kDa. Gel is comparing non-adhesive non-water hardened (N.W.H) chorions with adhesive water hardened (W.H.) chorions. The protein band present at approximately 30kDa within water hardened eggs is missing in non-water hardened eggs.

Table 7-1: Information of identified proteins in 1D gels by MS and bioinformatics.

Variable	Accession no.	Species of origin	Peptides matched
Chain A, RNase ZF-3e	gi190613445	*Danio rerio*	20
Translation initiation factor IF-2,mitochondrial precursor	gi209153992	*Salmo salar*	9
Vitellogenin	gi3688439	*Oncorhynchus mykiss*	2
Vitellogenin-1	gi88985004	*Fundulus heteroclitus*	2
Vitellogenin precursor	gi4572552	*Pimephales promelas*	3
Vitellogenin-5	gi68448530	*Danio rerio*	2
Vitellogenin	gi16151379	*Platichthys flesus*	1
Vitellogenin B	gi95114406	*Merluccius merluccius*	1
Vitellogenin	gi145904568	*Rhinichthys cataractae*	1
Vitellogenin II	gi157278415	*Oryzias latipes*	1

7.5 Discussion

The current study demonstrated that European whitefish eggs become adhesive, minutes after mixing with water, and that 30 seconds of contact with water was enough to initiate adhesion. These results are similar to previous studies on common carp (*Cyprinus carpio*) and Eurasian perch (*Perca fluviatilis*) eggs (Mansour *et al.*, 2009a; 2009b), which showed very rapid activation of adhesive properties. Results presented here also indicate that this adhesive property originates from the chorion and that both biochemical and mechanical mechanisms are involved. Many teleost species produce adhesive eggs, and it has been proposed that this mechanism enables females to deposit eggs onto optimal spawning environments and ensures that eggs remain there, which in turn increases the likelihood of their survival (Riehl and Patzner, 1998).

Unlike other species of salmonid which build nests, European whitefish are broadcast spawners, and both male and female gametes are randomly dispersed over the selected habitat. In the case of European whitefish, habitat comprises well-washed, shallow, littoral and sublittoral gravel banks, however, these areas are exposed, and therefore, subjected to substantial wave action (Brown and Scott, 1994; Low *et al.*, 2011).

The adhesiveness of European whitefish eggs failed to activate when immersed in 300mmol/l (=300 mosmol/kg) NaCl. Highly hypertonic solutions of NaCl have been used during previous studies to inhibit water hardening and thus the adhesive properties of eggs from other teleosts (Mansour *et al.*, 2009a). *In vivo* it is unlikely that ovarian osmolarity alone controls adhesion and that a synergistic response involving other factors such as temperature and pH are also involved (Mansour *et al.*, 2009). The ability to inhibit adhesion is of particular importance to the aquaculture industry especially for commercially expensive species such as the sturgeons (Family Acipenseridae). For example, the eggs of the white sturgeon (*Acipenser transmontanus*) adhere to the substrate, or each other, shortly after contact with fresh water (Monaco and Doroshov, 1983). Incubation of adhesive eggs is virtually impossible in standard incubators, due to high mortality caused by fungal growth. Therefore, methods such as those involving solutions which neutralise egg adhesion while allowing the egg to survive could further egg incubation and fish culturing techniques (Mansour *et al.*, 2009a).

Examination of scanning electron micrographs showed that the surface of the chorion altered considerably after water hardening. These results suggest that the nodule like protuberance may facilitate adhesion either by direct contact via interlocking protuberances, or by creating friction between the surface of the egg and the substrate onto which it falls. The nodule-like protuberances observed during this study are similar to the villi-like protuberances reported in the eggs of *Vimba vimba* (Riehl and Patzner, 1998). However, while the adhesive structures on the eggs of *V. vimba* were approximately 4μm in length, those on the eggs of European whitefish were both shorter and more varied (between 1μm and 3μm in length).

The ultra-structural observations of the chorion reported here showing irregularly spaced non-symmetrical protuberances are in direct contrast to those reported by Scapigliati *et al.*, (1995), who showed that the surface of non-water hardened *C. lavaretus* eggs was relatively smooth and clear of any protuberances. One possible explanation for these differences is the occurrence of phenotypic variation between populations of coregonids (Bernatchez *et al.*, 1999; Etheridge *et al.*, 2010). Differences in the structure of whitefish eggs from Loch Eck and those gathered by Scapigliati *et al.*, (1995) from Lake Bolsena may represent a variation in egg morphology between these populations.

From both previous studies and the results of this study it appears that that one or more proteins are involved in the adhesive properties of whitefish eggs. Histological examination of the chorions of adhesive water hardened eggs showed the ZRE staining positive for Alcian blue and PAS. These results are consistent with those found in other species of teleost which produce adhesive eggs (Mansour *et al.*, 2009a; 2009b; Riehl and Patzner, 1998). Glycoproteins have been identified as a family of proteins important for egg adhesion and the presence of the blue stain on the ZRE of Loch Eck whitefish eggs suggests that adhesive molecules originated from this section of the chorion. The chorion as a whole was composed of approximately 12 protein bands as detected by electrophoresis. A similar profile was observed in both water hardened and non-water hardened eggs, however, the concentration of proteins increased after water hardening. One band, located at approximately 30kDa, was not present in non-adhesive non water hardened chorions but was present in adhesive water hardened chorions. Analysis of the band in question resulted in the identification

of three likely molecules; chain A RNase ZF-3e, translation initiation factor IF-2 and vitellogenin (in decreasing order of peptides matched).

RNase ZF-3e belongs to the superfamily of pyrimidine-specific ribonucleases, many of which have known biological activities; for example, some stimulate the development of vascular endothelial cells, dendritic cells and neurons, others are cytotoxic/anti tumoral and/or anti-pathogenic (Kazakou *et al.*, 2008). An adhesive activity has yet to be identified within this enzyme family, however the structural diversity of this family of enzymes is varied and structural alterations are known to influence biological activity (Kazakou *et al.*, 2008). Alternatively, the presence of RNase ZF-3e may indicate the activation of immune factors on the chorion of water hardened eggs and may not have any role in egg adhesion. The maternal transfer of specific immune factors such as IgM, complement, lectins, protease inhibitors and lysozymes have been reported in other species of fish (Lovoll *et al.*, 2007; Mor and Avtalion, 1990; Takemura 1996; Tateno *et al.*, 2002). Yet the list of immune factors present and the mechanisms that allow these eggs to survive pathogens before the maturation of their own immunocompetence remains poorly understood (Wang and Zhang, 2010).

Initiation factor 2 (IF-2) is one of three factors (IF-3 and IF-4) required for the initiation of protein biosynthesis in eukaryotes (Lee *et al.*, 1999). IF-2 promotes the GTP-dependent binding of the initiator tRNA to the small subunit of the ribosome. This process takes place during the 'initiation phase' of mRNA's translation to protein (Lee *et al.*, 1999). The identification of this molecule in the protein profile of adhesive water hardened European whitefish eggs suggests that IF-2 was increased during the hardening process in order to manufacture the adhesive proteins, but was not directly involved in egg adhesion.

Vitellogenins are members of the Large Lipid Transfer Protein (LLTP) superfamily (Buisine *et al.*, 2002). Vitellogenin is the egg yolk precursor manufactured in the liver and transported to the ovary via the plasma during vitellogenesis (Arukwe and Goksoyr, 2003). Oocyte yolk proteins are formed by the enzymatic cleavage of vitellogenin and other very low density lipoproteins, into the yolk proteins (Lipovitellin and phosvitin) (Arukwe and Goksoyr, 2003). There are no known adhesive activities associated with this molecule and it is unlikely that it is involved in egg adhesion. It is more likely that this molecule originated from the

residual yolk which had attached itself to the chorion during the chorion isolation process.

Scottish populations of whitefish are currently under pressure from a number of biotic and abiotic factors; egg adhesion plays an important role in maintaining recruitment levels in this species (Slack *et al*., 1957). By spawning in high energy areas *C. lavaretus* ensure an adequate supply of well oxygenated water with little chance of sediment deposition on their eggs. Without adhesive properties these eggs may be physically damaged by wave action or washed ashore, dying from desiccation or disturbed into deeper water which is less well oxygenated and sediment loads are heavier.

8 General Discussion

8.1 Overview

The main objectives of the current study were to compare novel and previously examined methods of egg quality assessment in salmonids, and how changes to broodstock environment and nutrition affected egg quality in fish held under commercial conditions. It was also the aim of this study to examine the maternal transfer of dietary selenium (Se) to the eggs of farmed salmonids and determine the role of dietary supplementation on *Saprolegnia* resistance in farmed fish eggs. In addition, this study also began preliminary investigations into the structure and biochemical contents of the chorion of eggs from European whitefish (*Coregonus lavaretus*) from Loch Eck, focusing on how these biological and morphological modifications may produce adhesive properties.

The results of the present study indicate that energy-dispersive x-ray spectroscopy (EDX), chorion breaking strength measurements, protein analysis and alternative data analysis techniques can be applied along with egg survival measurements to provide an accurate understanding of egg quality. Furthermore, differences in broodstock holding environments and intra-population variation can cause significant variation in the quality of eggs produced by farmed salmonids. This study has also provided the first conclusive evidence of dietary Se being transferred from Atlantic salmon (*Salmo salar*) broodstock to their eggs during reproductive development. However, there is no conclusive evidence to suggest that dietary Se supplementation of Atlantic salmon affects the parameters of egg quality examined. The current study also identified the presence of adhesive mechanisms on the eggs of European whitefish which indicates that alterations to the morphological and biochemical structure of the chorion may be important in aiding egg survival in species which spawn in high energy environments. The following sections of this chapter will discuss these issues in more detail and suggest where future work must focus.

8.2 An assessment of the methods used to assess egg quality

In Chapter 2 this thesis used a variety of both previously researched and novel methods of fish egg assessment to identify characteristics of egg quality in brown trout (*Salmo trutta*). Subsequent analysis of the data identified egg survival, egg elemental analysis, chorion breaking strength and chorion protein profiling as the tests with the most potential for developing measures of egg quality for the duration of this study. Furthermore, due to the limited information available regarding how the constituents and characteristics of the chorion affects egg survival this thesis choose to focus on the chorions relationship with egg quality.

8.2.1 Egg survival as a measure of egg quality

Egg quality can be defined as an eggs ability to be fertilised, develop normally and subsequently hatch, therefore, egg survival measurements at these key developmental stages are often thought of as the most important parameter of egg quality and as such, are used routinely by the aquaculture industry (Bobe and Labbe, 2010). However, despite the obvious importance of egg survival data this measure fails to describe the characteristics of the egg which allowed it to reach these key developmental stages. Although routinely used within the commercial fish farming industry, under experimental conditions egg survival experiments are labour intensive and require constant observation. During this study the incubation environment was checked regularly in-order to ensure that water conditions were standardised between systems and dead eggs removed immediately to prevent pathogenic infection of healthy eggs (Theon *et al.*, 2011). Previous studies have found that eggs from individual chum salmon (*Oncorhynchus keta*) incubated at 4^{o}C, 8^{o}C and 12^{o}C had significantly different survival rates (Beachman and Murray, 1985). Results like these confirm that the incubation of fish eggs needs to be strictly controlled or it may result in erroneous results regarding egg survival rates. In order to accurately assess egg quality, studies need to concurrently examine the intrinsic properties of the egg and combine this data with egg survival measurements.

8.2.2 Egg elemental analysis as a measure of egg quality

There are many benefits to using EDX to analyse egg quality in farmed fish. For example, the concentration of any element within the yolk and chorion can be extrapolated using this method, which is particularly useful as many trace elementals are essential for normal biological growth (Watanabe *et al.*, 1997). Furthermore, under commercial aquaculture conditions, sample processing for EDX analysis was relatively flexible and easy to perform. Eggs were stored at low temperatures during this study, but may also be placed in a biological fixative or processed immediately depending on the situation (Echlin 2001). While EDX analysis has many benefits one of the major drawbacks to using this method of analysis is that the results are semi-quantitative and therefore not as accurate as alternative methods for analysing elemental concentrations (Arrigoni *et al.*, 2006). EDX is a good method for screening for a wide variety of elements and is beneficial for preliminary investigations into samples where the elemental constituents are unknown. However, when investigating the presence and concentration of a specific element, mass spectrometry provides more accurate and quantitative results.

8.2.3 Chorion breaking strength as a measure of egg quality

The chorion is a robust proteinaceous structure which surrounds fish eggs and protects the developing embryo within from a number of hazards including mechanical damage (Brooks *et al.*, 1997). In order to test the robustness of the chorion and its ability to resist mechanical damage, eggs were subjected to a chorion breaking strength procedure using a Lloyd LXR instrument, a machine designed for the food technology industry. Once a mechanism was constructed to hold the egg in place during the procedure the instrument was able to detect the pressure differences which occur during the breaking point of water hardened and non water hardened chorions. There was no significant benefit of testing water hardened over non-water hardened eggs, however the solution used to harden the chorion may affect the physical properties of the egg, and therefore further studies would ideally examine non-water hardened chorions only. Like EDX analysis, preparation for the chorion breaking strength procedure was straightforward however, eggs must be processed as soon as possible as any type of fixation solution is likely to alter the physical attributes of the chorion

and consequently provide misleading data concerning the true strength of this structure.

8.2.4 Chorion protein analysis as a measure of egg quality

A substantial amount of refinement was required in order to optimise for the identification of chorion proteins in the eggs of salmonids. During the initial study (Chapter 2) two difficulties were highlighted, specifically problems regarding the contamination of the chorion with leftover yolk proteins, and the sensitivity of the protein assay and 1D-gel electrophoresis to samples containing a minimum concentration of proteins.

Yolk protein contamination of chorion samples is problematic as it adds a wide range of high molecular weight proteins which aggregate within the gel plate during electrophoresis distorting the profile of the lower molecular weight proteins. Initial attempts to find a solution to this problem utilised a salt precipitation method based on a technique employed by Stanley *et al.*, (2002) to remove serum albumin from cellular extracts. While this provided satisfactory results, the method was time consuming and took approximately two full lab days to prepare a sample for protein assaying. Moreover, this procedure is not specific to yolk proteins. For example, other high MW proteins related to the chorion may also be removed unintentionally along with the yolk proteins.

An alternative to the salt precipitation technique involved washing the chorion in a weak concentration of Tris buffer prior to the protein assay and SDS-gel electrophoresis. Like the salt precipitation technique this procedure also provided protein profiles clear of yolk contamination, but added very little time (approximately 2 hours) onto the original procedure. This process ensured that the contamination was removed or minimised, while at the same time preventing the loss of other potentially significant proteins. These advantages resulted in this method being employed throughout the remainder of this study.

Due to the relatively small number of chorions (n=10) used from each individual fish, the sensitivity of the SDS-gel electrophoresis to low protein concentrations and to the presence of proteins with low molecular weights (MW) needed to be increased. This was performed using two adaptations to the initial protocol. The

first adaptation involved increasing the concentration of chorion proteins in solution for each individual sample using an acetone precipitation procedure (Stanley *et al.*, 2002). The second adaptation involved improving the sensitivity of the procedure using a silver stain derivative after the electrophoretic separation instead of a Coomassie blue stain. Previous studies have reported that the stoichiometric interaction between silver compounds, mainly silver nitrate or silver-ammonia complex solution, and protein is 10-50 fold more sensitive than standard Coomassie blue stains (Butcher and Tomkins, 1984; Oakley *et al.*, 1980; Willoughby and Lambert, 1983). These two modifications to the initial methodology allowed the clear profiling of proteins and the estimation of their concentrations using pixel density measurements.

An attempt was made to further develop the proteomic analysis during Chapter 5 using 2D-gel electrophoresis to separate bands containing multiple proteins within chorionic samples into single protein spots for identification. The difficulties experienced during 2D-gel electrophoresis procedures are thought to have originated from the samples becoming contaminated with salt during the initial processing steps, which is discussed in detail in Chapter 5. While samples prepared for 1D-gel electrophoresis are able to cope with highly ionic concentrations due to the absence of the electro-focusing step, samples for 2D-gel electrophoresis must be prepared differently in order to take into account the sensitivity of this procedure to salt. Therefore, chorions for 1D and 2D-gel electrophoresis should be processed separately with the samples intended for 2D gel-electrophoresis being homogenised in a lysis and low ionic rehydration buffer instead of the homogenisation buffer used throughout this thesis.

The current study has shown that proteomic analysis, specifically gel electrophoresis, is a sensitive technique able to detect subtle shifts in the presence and concentrations of specific proteins within the chorion. In order to examine the importance of these changes in relation to fish egg quality it is important to develop a species specific baseline of which proteins are present and what there function is. Once the presence and concentrations of the ‘major’ and ‘minor’ proteins within the chorion have been identified and their functions defined, it may be possible to use proteomic techniques to identify protein biomarkers of egg quality.

8.2.5 The use of multivariate analyses to define egg quality

The egg is a complex structure made from up from a number of components which themselves are constructed from wide variety of materials, and while the majority of mechanisms involved in its construction are well documented many of the physiological processes are still poorly understood (Tyler and Sumpter, 1996). Due to the complex nature of the egg, issues of egg quality are more likely to be explained by examining multiple egg parameters rather than focusing on a single parameter. The majority of studies to date have examined one or two parameters of egg quality at a time, few studies have attempted to examine multiple egg quality parameters (Lahnsteiner *et al*., 1999) and none recorded have attempted to integrate the data obtained. Chapter 3 examined the use of an alternative means of egg quality analysis in which a principle components analysis (PCA) was used to combine chorion EDX data and chorion breaking strength data into a single usable egg quality score per individual female. These scores were then used to examine the linear relationship between egg survival and thus egg quality. The results from Chapter 3 found that the PC scores explained a significant amount of variation in egg survival (40%) and that there was a linear relationship between the egg quality scores and egg survival. Other chapters examining the relationship between a single parameter of egg quality and egg survival did not find a significant relationship between these measures providing significant evidence towards the benefits of utilising multivariate data analysis techniques.

Chapter 3 focused solely on chorion quality parameters, however further investigations into the use of multivariate analyses to predict egg survival and thus egg quality would require a more integrated approach, such as incorporating parameters of the yolk and possibly other components of the egg as well. Combining chorion and yolk quality parameters into a single egg quality score would provide a more robust representation of egg quality and may increase the significance of the results. Classical linear models for regression and analysis of variance are widely used to examine the relationship between explanatory variables and response variables, with the development of alternative methods of data analysis it may eventually be possible to produce a standardised model of egg quality, which would potentially allow the

aquaculture industry to identify batches of eggs with poor survival potential earlier than currently possible.

8.3 Factors identified in this thesis as affecting egg quality in salmonids

In order to accurately assess egg quality parameters, factors which may influence reproductive success must be identified and their effects on the egg detailed. Previous studies have indicated that factors such as diet, environment, stress and genetics are all capable of influencing broodstock and subsequently affecting egg quality, however research concerning the specific effects these factors have on the characteristics of the egg remains limited.

8.3.1 Holding environment and egg quality

Chapters 3 investigated the effect of the holding environment and found that despite brown trout originating from the same strain and being fed the same diet; elemental yolk and chorion concentrations, chorion breaking strength and egg survival were all significantly affected by the different environments. The exact cause of these changes in egg quality is unknown as there were a number of differences between the holding environments. However, the significantly higher survival rate and burst strength of eggs produced by fish held at S.C.E.N.E. suggests that this environment increased egg quality compared to the facilities at Ae Fishery. Although the differences in holding environment may have been responsible for the results observed in Chapter 3, either individually or synergistically, it is also possible that the stress experienced by fish held under standard aquaculture conditions was the most important factor.

Various stressors such as anthropogenic interactions, holding densities, physio-chemical condition of the water and intra-specific competition are inexplicably linked to the modern aquaculture industry and may cause a decrease in reproductive capacity and subsequently egg quality (Iwama *et al.*, 2006). Studies have shown that stress in fish causes a change in whole body energy partitioning, resulting in energy being channelled away from reproduction and directed towards systems that enable the animal to cope with the enhanced energy demand associated with a stress response (Carragher *et al.*, 1989; Pottinger and

Pickering, 1990). At Ae Fishery, anthropogenic disturbances were common due to standard practice at this site which included hand feeding fish in adjacent tanks and generally monitoring fish stocks. Studies have shown that this form of high frequency low-level disturbance causes higher welfare costs to fish compared to other forms of husbandry (Adams *et al.*, 2007 Turnbull *et al.*, 2005). By monitoring the plasma cortisol concentrations of broodstock during reproductive development further investigations may be able to examine the relationship between the broodstock endocrine system and egg quality.

Another factor which may have affected the physiological status of the fish, and thus egg quality, in Chapter 3 was the hydrology within the holding systems. Although both populations experienced similar volumes of water passing through the tank the way water entered the tanks and raceways were dissimilar. Water entering the tanks at S.C.E.N.E entered side on from three pipes, positioned equidistantly around the circular holding system, this resulted in the formation of a current which the fish were regularly observed swimming against. Alternatively, water entering the raceway at Ae Fishery was supplied via an overhead pipe, which caused no such current to form. Increasing the current within a system causes the fish to swim against it and has been shown to decrease physiological and behavioural stress responses in farmed fish by exercising fish and reducing attacks by con-specifics (Adams *et al.*, 1995; Cutts *et al.*, 1998; Damsgard and Arnesen, 1998). The current within the tanks at S.C.E.N.E. may therefore have allowed broodstock to devote more energy to reproduction and subsequently increased the quality of eggs produced. Studies must investigate the effect of broodstock holding tank hydrology on egg quality by rearing farmed fish in tanks with and without currents and examining the effect this environment has on the intrinsic properties of the eggs and egg survival.

8.3.2 Inter-female variation and egg quality

After confirming that holding environment did affect egg quality Chapter 4 removed ‘environment’ as a factor by holding all fish within the same system and examined intra-population variation in egg quality under commercial production conditions. Despite the holding environment and diet being identical for all broodstock sampled, the results showed that there was a large variation

in not only egg survival rates, but also chorion breaking strength, chorion protein profiles and egg elemental concentrations between individuals. Although extrinsic effects on egg quality are more common and as such, a focus of current research (Bobe and Labbe, 2010), these results strongly suggest that more consideration must be given to intrinsic factors such as the genetics of the adult broodstock when examining egg quality. Stoddard *et al.*, (2005) suggested that early onset embryonic mortality of some rainbow trout eggs was caused by the insufficient transfer of important maternal genotypes to the egg during development. The hierarchal structure and social status of individual farmed fish, a function of their behaviour, is also likely to affect egg quality through the complex interactions between this type of conduct and physiological responses associated with reproduction (Bobe and Labbe, 2010; Iwama *et al.*, 2006). Both intrinsic factors are likely to be important to the commercial aquaculture industry, but both topics remain poorly documented.

8.4 The effect of dietary Se supplementation on egg quality in salmonids

In Chapter 5, Atlantic salmon given an industrial feed supplemented with 0.5mg/kg of Sel-plex produced eggs with significantly higher Se concentrations than those fed the non-supplemented industrial diet. As each individual broodfish was held in tanks containing water from the same source this indicates that dietary Se supplementation and not Se within the water column, as stated during previous studies (Coyle *et al.*, 1993; Schultz and Heramntuz, 1990), was responsible for the increased concentrations of this element within the eggs produced by fish fed the supplemented diet. Additional investigations are required to examine the maternal transfer of Se within the diet to the yolk of developing oocytes. While Chapter 5 indicates that Se supplemented within the broodstock diet is transferred to the eggs of Atlantic salmon, it does not describe in what form the Se is present and in what locations is it distributed within the egg remains unknown. The most important outcome of any study in this area must be to both identify what biological form Se takes and what is its physiological importance.

The chorion of teleosts eggs has anti-pathogenic functions and previous studies have shown that chorion extracts from fish eggs exerted an anti-pathogenic

action on *Saprolegnia parasitica* (Kudo and Teshima, 1991). It is also known that disease resistance in salmonids is compromised by Se deficiencies (Thorarinsson *et al.*, 1994), therefore it was suggested that Se supplementation of broodstock diets may improve disease resistance in their eggs. When it became apparent that the Se concentration in the eggs of Atlantic salmon fed the supplemented diet had increased (Chapter 5), a sub-sample of the eggs produced were tested for their ability to resist saprolegnosis (Chapter 6). Ultimately the results showed no significant difference between the *Saprolegnia* resistance of eggs from fish fed a Se supplemented diet compared to eggs from fish fed the non-supplemented diet. However, this study focused on the chorions ability to resist saprolegnosis but this disease also affects juvenile fish after hatching. If Se is stored within the yolk instead of the chorion, as found during studies in poultry eggs (Surai *et al.*, 2004), then the benefits of dietary Se supplementation to the immune system of fish may extend beyond these early stages of embryonic development.

8.5 Physical and biochemical modifications of the teleost chorion

The results from Chapter 7 highlighted the presence of previously undocumented adhesive properties in the eggs of European whitefish. The ability of some species of fish to produce adhesive eggs has been attributed to the selection of spawning grounds that increases the likelihood of egg survival and thus recruitment to the resident population (Riehl and Patzner, 1998). In terms of the European whitefish populations in Loch Eck, the discovery that these fish produce adhesive eggs (Chapter 7) may ensure that eggs remain in high energy, shallow offshore gravel banks where the eggs are well aerated. Without adhesive mechanisms these eggs may be damaged outright or transferred, by wave-action, to sub-optimal hatchery areas were there is an increased risk of mortality (Mansour *et al.*, 2009b).

The chorion from the eggs of European whitefish produced a strong positive reaction for both PAS and Alcian blue stains. These results are similar to those reported in fish eggs containing a biochemical adhesive layer. For example, the chorion of pike eggs also produced a strong PAS reaction, while a study on the adhesive properties of common carp eggs showed that the chorion produced a

strong reaction to PAS as well as Alcian blue (Mansour *et al.*, 2009b; Riehl and Schulte, 1977). Unusually, the results from Chapter 7 also suggest that European whitefish eggs contain an addition adhesive mechanism as they also exhibited physical characteristics consistent with mechanical attachment mechanisms. High magnification microscopy techniques identified the presence of non-symmetrical villi-like protuberances irregularly spaced around the surf of eggs from this species (Chapter 7), similar in design as the attachment mechanisms indentified on the eggs of vimba bream (*Vimba vimba*) (Riehl and Patzner, 1998).

The presence of adhesive mechanisms on fish eggs and the underlying mechanisms which produce this activity remain poorly understood, however, available reports suggest that the classification of two types of adhesive mechanisms on eggs form a single species is unusual. While Riehl and Patzner, (1998) indicated that a number of species of substrate spawning cichlids (*Cichlasoma*, *Geophagus*, *Hemichromis*, *Pterophtylum* and *Symphysodon*) produced eggs with attaching filaments and an adhesive layer, Meijide and Guerrero, (2000) stated that eggs from *Cichlasoma* species are stuck to the surface of the substrate with an adhesive mucous layer or attachment filaments, not both. Further work is required to determine exactly what bio-molecules are responsible for the adhesive layer and why such mechanisms appear to be present on some populations of European whitefish and not others.

8.6 Future work

8.6.1 The use of proteomic techniques to define egg quality in salmonids.

Considering the importance of variations in egg quality to the aquaculture industry, the biological importance of the chorion to embryogenesis and the potential of proteomic analysis, it is surprising that information within the literature concerning the identity and function of fish chorion proteins and their use as biomarkers for egg quality is fragmentary and limited. A multitude of information concerning the identification and function of chorion proteins has been added to recently (Arukwe and Goksoyr, 2003; Modig *et al.*, 2007). In part this has been aided by the close phylogenetic relationship between mammalian

and teleost eggshell proteins (Epifano *et al.*, 1995; Oppen-Bernsten 1999), however inter species differences in the physiology of the egg has produced alternative complexities in the macromolecular composition between fish and mammalian eggs (Modig *et al.*, 2007). Furthermore, recent studies have highlighted that despite the conserved nature of the chorion proteins, there exist sufficient differences to suggest that identification of proteins within the chorion and the selection of protein biomarkers for egg quality should not generalised as a teleost model but be species specific instead (Griffin *et al.*, 1996; Mansour *et al.*, 2009a; Modig *et al.*, 2007). In order to obtain more detailed knowledge as to why there is a significant amount of variation in egg survival rates within farmed fish species, more sensitive and reproducible egg quality parameters are required. Chorion proteins may fill both of these requirements and provide more reliable biomarkers of egg quality at earlier stages of development compared to some parameters currently utilised within the aquaculture industry (Bobe and Labbe, 2010; Brooks *et al.*, 1997).

8.6.2 The effect individual broodstock genetics has on egg quality.

The results from Chapter 4 show that in spite of fish being fed the same diet and held under the same environmental conditions, there was a large amount of variation in recorded egg quality parameters. This result strongly indicates that maternal genes, or non-yolky cytoplasmic components, which accumulate during oocyte production, may be responsible for the observed variation (Bobe and Labbe, 2010; Brooks *et al.*, 1997). The earliest steps of embryogenesis are derived from maternal factors produced during oogenesis and stored within the oocyte in the form of messenger RNA (mRNA) and protein (Lyman-Gingerich and Pelegri, 2007). Despite the obvious importance of molecules such as mRNA, few studies have investigated their relationship to egg quality (Aegerter *et al.*, 2005; Bobe and Labbe, 2010). Most of the information available regarding the role of maternal mRNA during embryogenesis in oviparous vertebrates comes from work undertaken on amphibians and zebrafish (*Danio rerio*) (Brooks *et al.*, 1997; Bobe and Labbe, 2010; Lyman-Gingerich and Pelegri, 2007). However, there are fundamental differences in early embryonic development between classes and species (Baumann and Sander, 1984; Lyman-Gingerich and Pelegri, 2007; Nagler 2000; Stroband *et al.*, 1992), therefore, future studies must take into account

the specificity of the species when attempting to identify mRNA transcripts and their effect on egg survival and thus egg quality.

8.6.3 Determination of dietary Se requirements for broodstock salmonids

The importance of trace elements, like Se, to the health and development of farmed fish is a relatively recent research priority, and as such information regarding key issues is either limited or requires additional investigations. Despite the latest studies indicating that organic trace elements are potentially less toxic to fish compared to inorganic forms, the majority of studies using dietary Se supplements have focused on the inorganic forms selenite, selenate and selenide (Hilton *et al.*, 1980; Rider *et al.*, 2009; Rider *et al.*, 2010). However, it is clear from previous studies that the uptake mechanisms, storage capabilities and metabolic processes of inorganic trace elements is different to those that are organically bound (Rider *et al.*, 2009). The positive effect of dietary trace elements in other domesticated animals, such as poultry, pigs and cows, highlights the need for the metabolism and utilisation of organic Se in fish to be characterised and documented (Surai 2006; Rider *et al.*, 2009).

One of the fundamental problems with the current determination of Se requirements in the diet of farmed fish is that it is based on the nutritional needs of rapidly growing juveniles which does not take into account the additional physiological needs of fish bred for their reproductive qualities. A number of studies indicate that additional concentrations of bulk dietary components such as proteins, lipids and carbohydrates are needed to meet the increased energy demands placed on broodstock during sexual development, however, the nutritional requirements for many of the so-called ‘minor dietary constituents’, such as Se, remain unclear (Brooks *et al.*, 1997; Fernandez-Palacios *et al.*, 1997; Izquierdo *et al.*, 2001; Luquet and Watanabe, 1986). In order to fully exploit the potential benefits of Se, further studies need to investigate the appropriate concentration of dietary Se required so that the health of the adult broodstock is enhanced as is the quality of eggs that they are able to produce.

In order to establish the optimal level of dietary Se for broodstock and determine its effect on egg quality, studies also require physiological markers to accurately assess the nutrition and mineral status of Se in fish and eggs. The majority of studies have used the activity of the selenoprotein, glutathione peroxidase (GSH-Px), to investigate the concentration and bioavailability of Se within mammals, birds and fish (Surai 2006; Hilton *et al.*, 1980). However, there are a number of limiting factors, such as biological activity and availability, which prevent GSH-Px from being the ideal measure of Se status (Behne and Wolters, 1983; Berggren *et al.*, 1999; Brown and Arthur 2001; Burk and Hill, 2005; Hilton *et al.*, 1980; Neve, 2000). Therefore, a general investigation examining the expression and activity of all known selenoproteins in broodstock fed organic Se-supplemented diets would be able to identify a relevant marker for optimal biological activity in fish eggs.

8.6.4 The effect of broodstock Se supplementation pre and post hatching

This study mainly focused on the effect Se supplementation of salmonid broodstock diets had on the chorion quality parameters of the egg. However, while this structure is essential for protecting the developing embryo against mechanical and environmental stressors, other components of the egg are directly involved in embryogenesis (Tyler and Sumpter, 1996). For example, the yolk contains various macromolecules (proteins, nucleic acids, carbohydrates and lipids) and micronutrients (vitamins, zinc, iron and selenium) which provide the necessary materials to form the embryo and physiologically drive development (Brooks *et al.*, 1997). In normal physiological conditions there is a continuous production of reactive oxygen species (ROS) which induce oxidative stress and can result in breaking down cell membranes, inactivation of enzymes and damage to genetic material (Mourente *et al.*, 1999). Previous studies have shown that the anti-oxidant selenoprotein GSH-Px is present in fish eggs post-activation and within the yolk-sac post-hatching (Barton 2002; Cowey *et al.*, 1985; Knox *et al.*, 1988). Future studies must endeavour to identify whether supplementing the diet of broodstock with Se increases the levels GSH-Px within the egg and/or benefits the embryo or juvenile fish by lowering the concentrations of ROS present.

8.7 Conclusions

Defining parameters of egg quality continues to be a contentious subject to those investigating this area of reproductive biology. In part this is due to different studies using different definitions of egg quality. However, one of the main problems with defining egg quality is the multitude of factors which may influence the intrinsic properties of the egg both pre and post ovulation. Everything from the species of teleost used during the experiment to the environment in which the broodstock and/or eggs are held has been shown to affect egg quality. This has made comparative studies difficult and resulted in the majority of studies having to be treated as stand-alone investigations.

The current study has found that chorion breaking strength and the concentration of certain elements within the chorion, analysed using energy dispersive x-ray spectroscopy (EDX) can be used to distinguish between eggs with high and low survival rates in certain salmonids species. Furthermore, proteomic analysis of the chorion shows considerable potential in identifying protein biomarkers of salmonid egg quality. This thesis has also found that broodstock holding environment significantly alters egg quality parameters and egg survival rates. However, even when broodstock holding environment and egg incubation environment is controlled, this thesis has shown that there is a large amount of variation in quality parameters of eggs from individuals, suggesting that maternal influences play a significant role in determining egg quality.

The results of the dietary investigations, regarding the use of Se-plex as a nutritional supplement of broodstock diet in order to increase egg quality is inconclusive. The initial trial on brown trout egg's showed that some parameters of the egg, such as lipid droplet distribution, elemental concentrations in the chorion and fecundity were affected by Sel-plex supplementation. However, due to significant environmental differences between replicate sites these results are unconvincing. The large scale industrial trial, held at Landcatch Ormsary found indications that Se supplemented into the diet of broodstock Atlantic salmon was maternally transferred to the eggs. This investigation has also shown that survival rates of eggs decreased when broodstock were given a supplemented diet compared to cohorts fed a non-supplemented diet and the chorion protein profile was also altered. While the supplemented diet contained

an additionally quantified source of Se, this was incorporated into nutritional matrix which contained additional concentrations of protein, lipid and carbohydrate. Due to the commercially sensitive nature of the diet this thesis was unable to categorically determine the constituents of the diet and state whether or not Se was the cause of the differences in egg quality parameters.

Finally, this thesis has identified previous un-recorded physical and biochemical adhesive mechanisms on the water hardened eggs produced by a population of European whitefish in Loch Eck. Eggs produced by these fish have both an adhesive layer and mechanical attachment mechanisms in the form of irregularly shaped protuberances, unevenly distributed around the surface of the chorion. The results also suggest that the adhesive layer originates from within the chorion and is produced by one or more proteins.

A Effect of selenium supplementation on egg quality in brown trout (*Salmo trutta*).

A.1 Introduction

Broodstock nutrition is an important factor likely to affect not only fecundity but also egg quality; however broodstock diet is without a doubt one of the most poorly understood and researched areas of finfish nutrition (Izquierdo *et al.*, 2001). Previous studies have highlighted that dietary component's as diverse as lipids, fatty acids, protein and absorbic acid can all affect egg quality (Brooks *et al.*, 1997; Izquierdo *et al.*, 2001). The variety of dietary constituents, which may affect egg quality, demonstrates how important even individual nutritional components are. Despite the need of detailed dietary analysis, information regarding the availability of trace elements such as zinc (Zn) and selenium (Se) and the affect that these so called 'minor' dietary constituents have on egg quality is negligible (Brooks *et al.*, 1997).

In recent years there has been particular interest in the role of the trace element selenium (Se) in the maintenance and promotion of animal health (Ganther 1999, Rayman 2000, Rider *et al.*, 2009 and Surai 2006) However, the role of Se on certain aspects of fish reproduction is largely unknown. The aim of this study was to determine the effect of Sel-plex®, an organic Se dietary supplement, on the egg quality of farmed brown trout (*Salmo trutta*) using the analytical methods developed in Chapter 2.

A.2 Materials and Methods

Forty brown trout (*Salmo trutta*) from the Ae Fishery, Dumfries, were used in this study. All fish were from the same strain. Twenty trout were transported to aquarium facilities at the Scottish Centre of Ecology and the Natural Environment (S.C.E.N.E.) University of Glasgow and held at these facilities from June 2008 until February 2009. Another 20 trout were held at Ae Fishery from June 2008 until November 2008.

A.2.1 S.C.E.N.E. broodstock holding environment

Broodstock were held in two 800L round, polyethylene tanks with a continuous supply of water (ca. 25L/min) from Loch Lomond, provided by three inlet pipes equidistant around the side of each tank. Waste water was drained using a central stand-pipe covered by a 5mm mesh screen. Fish were exposed to ambient Loch Lomond water temperature and photoperiod, delivered by artificial fluorescent lighting adjusted weekly to reflect natural photoperiod (Latitude =56.1403^{o}N). Fish within the control tank (Ctrl) were fed industrial food pellets (EWOS Ltd), which were dispensed daily by a clockwork belt feeder (Dryden Aqua Ltd) over a 24hr feeding period. Fish within the experimental tank (Exp) were fed the industrial food pellets supplemented with Sel-plex (Alltech Ltd). The amount of feed dispensed was over the recommended rate for the biomass of salmonids. Water temperature for the duration of the experiment ranged from 6^{o}C to 15^{o}C (mean=11^{o}C).

A.2.2 Ae Fishery broodstock holding environment

Broodstock were held in two 1000L raceways in a building at Ae Fishery. Both ends of the building were open sided and the roof comprised a transparent acrylic sheet, thus female broodstock were exposed to a natural photoperiod (latitude =55.1876^{o}N) with no artificial light sources. Water, drawn from the River Ae, entered the raceway from a pipe at one end of the raceway (ca. 30L/min), while waste water was drained by a standpipe screened by 5mm mesh located at the other end of the raceway. Control fish were fed industrial food pellets (EWOS Ltd), which were deposited into the tank by a clockwork belt feeder (Dryden Aqua Ltd) set to a 24hr continual feeding regime. Experimental fish were fed the same industrial food pellets supplemented with Sel-plex (Alltech Ltd). The amount of feed dispensed was above the recommended weight based on the biomass and recommended feeding rate for salmonids. Water temperature tracked ambient for the River Ae, ranging from 1^{o}C to 20^{o}C (mean= 11^{o}C).

A.2.3 Assessing reproductive status of broodstock

Reproductive maturation in individual trout was assessed, between October 2008 and February 2009, by anaesthetising fish in a benzocaine solution (Sigma Life

Sciences) and checking visually for signs of abdominal distension and egg release. Broodstock that were not ovulating were placed in a 150L recovery tank, before being returned to the holding tank. Ovulating fish were killed by exposure to a lethal dose of anaesthetic, followed by a sharp blow to the head (Schedule 1 method). Fish were blotted dry and their eggs were stripped into clean dry plastic tubs by abdominal manipulation.

A.2.4 Egg Survival

The details of how egg survival was determined out can be found in Chapter 2, section 1.2. In brief, 500 eggs from each female were sub-divided into two replicates, fertilised, activated and stored in individual incubation trays at S.C.E.N.E. The eggs were checked every alternative day for mortalities and any dead eggs found were recorded and removed from the incubation system.

A.2.5 Chorion breaking strength

In brief, chorion breaking strength was measured using 10 non-activated and ten activated eggs, collected from each individual female and tested using a Lloyd LRX compression test instrument. Details of this measure can be found in Chapter 2, section 1.3

A.2.6 Egg chorion and yolk element concentrations

The details of how egg chorion and yolk element concentrations were measured can be found in Chapter 2, section 1.4. In brief, chorions from 4 non-activated eggs, taken from each individual female, were removed then oven dried and mounted onto sticky tabs. For yolk elemental analysis, 4 additional eggs were dried, then cut in halve so that the yolk filled centre of the egg was exposed before being mounted onto carbon tabs. Analysis of the chorion elemental concentrations was carried out by energy-dispersive x-ray spectroscopy and Carbon (C), oxygen (O), sodium (Na), magnesium (Mg), phosphorus (P), sulphur (S), chlorine (Cl), potassium (K), calcium (Ca) and selenium (Se) were all consistently detected during analysis. The percentage concentration for each of these elements within the both the yolk and chorion were recorded

A.2.7 Fish Fecundity

Fecundity of each female brown trout was determined using the total weight of all ovulated eggs and an estimate of the number of individual eggs present. In brief, all eggs produced by an individual female were drained of ovarian fluid and weighed to provide the total weight of eggs produced. Ten eggs were then weighed and the number of eggs was estimated using the weight of 10 eggs calculated against the weight of the entire egg mass. Details of this procedure can be found in Chapter 2, section 1.5

A.2.8 Egg weight and diameter pre and post activation

In brief, ten eggs from each individual female were cleared of excess ovarian fluid. Each egg was weighed and its diameter measured before being placed in 100ml of water for 3hrs. Egg samples were then removed from the water and the weight and diameter was re-measured. The details of how the weight and diameters of eggs were measured can be found in Chapter 2, section 1.6.

A.2.9 Chorion protein analysis using SDS gel electrophoresis

The details of how the chorion was processed for SDS gel electrophoresis can be found in Chapter 2, section 1.7. In brief, chorions from 10 non-activated eggs, from each individual female, were removed and macerated in homogenisation buffer. The chorion protein concentration for the pooled sample was measured using a modified version of the Lowry assay (Lowry *et al.*, 1951) after which both concentrated (10µg/ml of protein) and dilute (1µg/ml of protein) chorion samples were prepared and run for protein profiling via SDS gel electrophoresis. Afterwards, gels were stained with a modified Coomassie blue stain and scanned onto a computer

A.2.10 Lipid droplet assessment

Ten non-activated eggs from each individual female were photographed at both poles and the lipid droplet distribution classified into categories as defined by Mansour *et al.*, (2007) by three independent assessors. Details of this procedure can be found in Chapter 2, section 1.8.

A.2.11 Data analysis

All data presented as percentages were arcsine transformed before statistical analysis and data collected from Ae Fishery and S.C.E.N.E. were examined independently. The effect of Sel-plex supplementation on egg quality variables with continuous data was examined using one way analysis of variance (ANOVA). The categorical data collected for the assessment of lipid droplet distributions were analysed using Kruskal-Wallis test. Regression analysis was used to examine the relationship between egg survival and the majority of egg quality parameters for eggs from fish fed the control diet only, experimental diets may alter egg parameters, therefore data collected from experimental eggs were not used during this analysis. Spearman rank order correlation was used to analyse the relationship between lipid droplet distributions and egg survival. Minitab® 16 was used to analyse the data.

A.3 Results

A.3.1 Elemental concentrations in the yolk and chorion of brown trout

The concentration of Se in the chorions of eggs produced by trout fed the experimental diet were significantly higher than those fed the control diet at Ae Fishery ($F_{[1,15]}$= 5.58, r^2= 0.29, p= 0.003) (Table A-1). Concentrations of S in the yolk of egg produced by trout fed the experimental diet were significantly lower compared to the trout fed the control diet ($F_{[1,15]}$= 5.42, r^2= 0.28, p= 0.035) (Table A-2). At S.C.E.N.E. trout fed the experimental diet produced egg containing lower concentrations of Se compared to the trout fed the control diet ($F_{[1,20]}$= 4.53, r^2= 0.19, p= 0.047) (Table A-2).

All other elements showed no significant difference in concentrations between trout fed the control or experimental diets (Table A-1; Table A-2).

Table A-1: Element concentrations (%) in the chorion of eggs produced by brown trout fed non-supplemented (Ctrl) and Se- supplemented diets (Exp).

Element (%)	Ae Fishery		Ae p-value	SCENE		SCENE p-value
	Exp (mn ± Std dev)	Ctrl (Mn ± Std dev)		Exp (Mn ± Std dev)	Ctrl (Mn ± Std dev)	
Se	4.14±0.58	2.60±1.85	**0.033**	4.37±0.60	4.10±0.87	0.418
C	47.59±3.10	48.77±1.80	0.386	47.96±1.41	47.70±2.67	0.776
O	38.83±3.05	38.43±2.52	0.784	39.10±2.16	39.55±2.14	0.638
Na	4.61±0.58	4.34±0.70	0.424	4.89±0.50	4.50±0.75	0.174
Mg	4.01±0.52	5.63±4.59	0.308	3.95±0.42	3.73±0.68	0.377
P	4.52±0.82	5.13±1.68	0.354	4.31±1.44	3.67±0.64	0.211
S	4.45±1.78	4.92±1.76	0.609	5.06±1.96	4.12±1.61	0.245
Cl	4.01±2.67	3.39±1.13	0.576	3.43±1.55	3.57±4.22	0.923
K	3.40±1.09	4.15±1.38	0.243	3.03±1.40	2.19±1.01	0.133
Ca	3.54±2.61	3.70±1.50	0.890	2.68±1.18	3.00±3.16	0.754

Table A-2: Element concentrations (%) in the yolk of eggs produced by brown trout fed non-supplemented (Ctrl) and Se-supplemented (Exp) diets.

Element (%)	Ae Fishery		Ae p-value	SCENE		SCENE p-value
	Exp (Mn ± Std dev)	Ctrl (Mn ± std dev)		Exp (Mn ± std dev)	Ctrl (Mn ± Std dev)	
Se	4.43±0.94	3.48±2.47	0.302	3.98±0.65	4.71±0.91	**0.047**
C	51.02±3.17	50.93±2.88	0.955	50.14±1.63	51.35±2.72	0.226
O	33.99±3.88	33.52±3.36	0.806	36.08±1.98	33.95±3.30	0.086
Na	4.61±0.52	4.50±0.80	0.745	4.21±0.57	4.50±0.30	0.168
Mg	3.92±0.54	4.46±0.64	0.093	3.58±0.54	4.03±0.45	0.053
P	6.47±2.04	7.38±1.75	0.362	5.74±1.96	6.05±1.37	0.674
S	6.08±1.22	7.84±1.81	**0.035**	5.79±2.30	6.15±1.63	0.685
Cl	5.13±2.18	5.61±1.83	0.653	4.22±1.97	4.82±2.00	0.502
K	6.62±1.89	6.81±2.40	0.243	5.43±2.79	5.31±1.81	0.906
Ca	4.37±1.70	4.47±0.95	0.893	3.26±1.85	3.64±1.36	0.592

A.3.2 Lipid droplet distribution in eggs produced by brown trout

Trout held at Ae Fishery and fed the experimental diet produced eggs with a higher yolk distribution score compared to those fed the control diet (H= 12.28, d.f.= 1, p< 0.001) (Table A-3).

There was no significant difference in lipid droplet distribution between trout held at S.C.E.N.E. and fed either control or experimental diets (Table A-3).

Table A-3: Lipid droplet distribution scores of brown trout fed non-supplemented (Ctrl) and Se-supplemented (Exp) diets.

Location	Exp Median Score	Ctrl Median Score	P-value
River Ae	1.75	2.38	>0.001
S.C.E.N.E.	2.00	1.75	0.281

A.3.3 Fecundity measurements of brown trout

Trout held at Ae Fishery and fed the control diet produced a larger volume of eggs compared to trout fed the experimental ($F_{[1,15]}$= 4.86, r^2= 0.26, p= 0.045) (Table 4) but there was no difference in the fecundity using the estimated number of eggs produced between dietary groups (Table A-4).

There was no significant difference in the fecundity (egg weight or the estimated number of eggs produced) by trout held at S.C.E.N.E. and fed either control or experimental diets (Table A-4).

Table A-4: Weight of eggs and estimated number of eggs of brown trout fed non-supplemented (Ctrl) and Se supplemented (Exp) diets.

Parameter	Ae Fishery		Ae p-value	SCENE		SCENE p-value
	Exp (Mn ± Std dev)	Ctrl (Mn ± Std dev)		Exp (Mn ± Std dev)	Ctrl (Mn ± Std dev)	
Fecundity in weight (g)	147.19 ± 30.95	192.09 ± 50.34	0.045	186.17 ± 63.65	221.85 ± 82.25	0.255
Fecundity by N° of eggs (est)	2583.8 ± 397.4	2902.4 ± 652.2	0.189	2711 ± 828	3296 ± 1215	0.189

A.3.4 Egg weight and diameter pre and post activation

Eggs produced by brown trout held at S.C.E.N.E. and fed the exp diet where significantly larger in diameter, when activated, compared to eggs produced by trout fed the ctrl diet ($F_{[1,20]}$= 11.40, r^2= 0.38, p = 0.003)(Table 5). There was no significant difference in the weight of pre or post-activated eggs, from trout held at S.C.E.N.E. (Table A-5).

There was no significant difference in diameter or weight of pre and post-activated eggs from trout held at Ae Fishery, (Table A-5).

Table A-5: Diameter and weight of eggs before and after activation in eggs from brown trout fed non-supplemented (Ctrl) and Se-supplemented (Exp) diets.

Parameter	Ae Fishery		Ae p-value	SCENE		SCENE p-value
	Exp (Mn ± Std dev)	Ctrl (Mn ± Std dev)		Exp (Mn ± Std dev)	Ctrl (Mn ± Std dev)	
Pre-activation diameter (mm)	4.53±0.36	4.49±0.20	0.809	4.81±0.39	4.66±0.31	0.320
Post activation diameter (mm)	4.92±0.27	4.50±0.19	0.524	5.40±0.29	5.18±0.17	0.003
Pre-activation weight (g)	0.06±0.01	0.06±0.01	0.704	0.07±0.01	0.06±0.01	0.359
Post activation weight (g)	0.07±0.02	0.07±0.01	0.601	0.08±0.01	0.08±0.02	0.692

A.3.5 Egg survival

There was no significant difference in the survival rates of eggs produced by trout held at Ae Fishery or S.C.E.N.E. and fed ctrl or exp diets (Table A-6).

Table A-6: Egg survival rates (%) in eggs produced by brown trout fed non supplemented (Ctrl) and Se-supplemented (Exp) diets.

Location	Exp (Mn ± Std dev)	Ctrl (Mn ± Std dev)	P-value
River Ae	20.62±17.96	17.42±19.55	0.739
S.C.E.N.E.	58.19±4.91	58.57±10.55	0.915

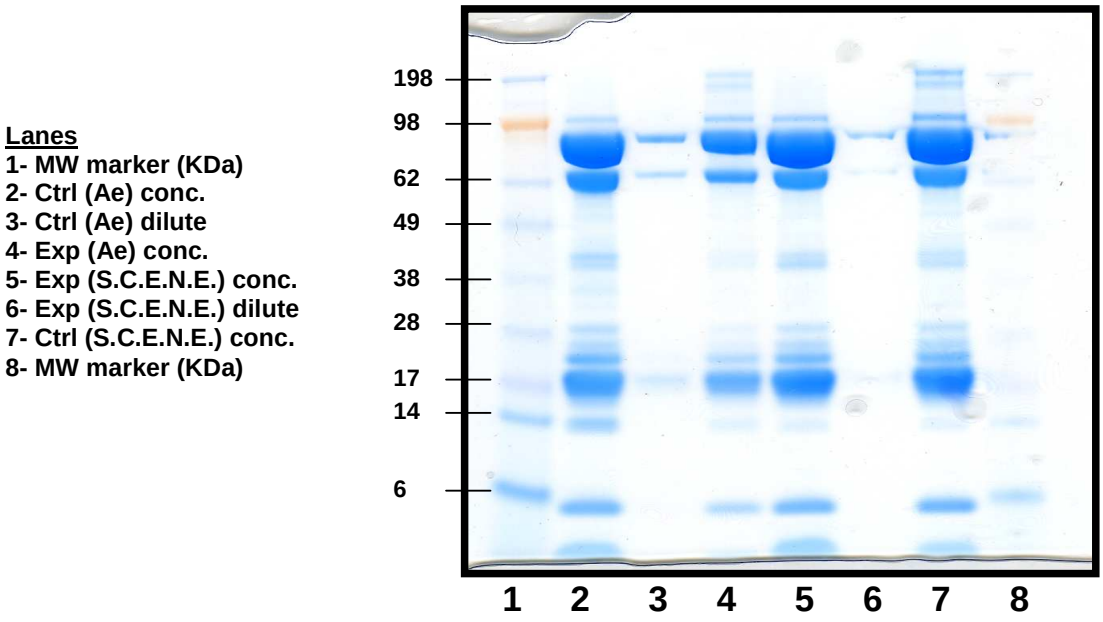

Figure A-1: SDS-gel electrophoresis showing examples of the neat (10µg/ml) and dilute (1µg/ml) chorion protein profiles of eggs from brown trout fed the non-supplemented diet (Ctrl) and the Se- supplemented (Exp) diet.

A.3.6 Chorion protein profile of brown trout eggs

Fourteen protein bands in total were identified on the gels at approximately 200, 198, 100, 98, 70, 41, 40, 27, 25, 24, 17, 14, 5 and 1KDa. An example of a typical protein profile of chorions from trout fed the ctrl and exp diet can be found in Figure A-1. Neat samples are heavily contaminated with albumen (approximately 90KDa) and dilute samples lacked sufficient protein concentrations to stain low molecular weight proteins (Figure A-1). Given these difficulties the results are inconclusive therefore at this stage no obvious differences existed between exp and ctrl diets at either S.C.E.N.E. or Ae Fishery

A.3.7 Chorion breaking strength measurements

There was no significant difference between the chorion breaking strength measurements of eggs from broodstock fed the experimental and control diet. The measurements obtained were comparable to the control diet at each site (Table A-7).

Table A-7: Chorion breaking strength of eggs produced by brown trout fed non-supplemented (Ctrl) and Se-supplemented (Exp) diets.

Parameter	Ae Fishery		Ae p-value	SCENE		SCENE p-value
	Exp (Mn ± Std dev)	Ctrl (Mn ± Std dev)		Exp (Mn ± Std dev)	Ctrl (Mn ± Std dev)	
Non-activated (N)	0.66±0.49	0.68±0.63	0.960	6.21±3.02	6.77±0.95	0.564
Activated (N)	1.18±0.26	1.38±1.35	0.659	17.87±8.15	20.06±4.09	0.437

A.3.8 Relationship between individual egg quality parameters and egg survival

For fish held at Ae Fishery there was a positive relationship between the breaking strength of non-activated chorions and egg survival ($F_{[1,6]}$=8.57, r^2= 0.63, p= 0.003), however there was also a strong negative relationship between the weight of the eggs produced and egg survival ($F_{[1,6]}$= 9.90, r^2= 0.66, p= 0.025) and sodium yolk concentrations and egg survival ($F_{[1,6]}$= 8.67, r^2= 0.63, p= 0.032) (Table A-8).

There were no significant relationships between egg quality parameters and egg survival for fish held at S.C.E.N.E. (Table A-8).

Table A-8: Relationship between parameters of egg quality and egg survival from a single group of brown trout held at Ae Fishery or S.C.E.N.E.

Parameter of egg quality v Egg survival	Ae Fishery p-value	SCENE p-value
Non-activated breaking strength (N)	**0.003**	0.650
Activated breaking strength (N)	0.083	0.074
Pre-activation diameter (mm)	0.920	0.763
Post activation diameter (mm)	0.309	0.536
Pre-activation weight (g)	0.190	0.234
Post activation weight (g)	0.127	0.761
Egg weight (g)	**0.025**	0.297
N° of eggs (est)	0.711	0.155
Se yolk (%)	0.288	0.359
C yolk (%)	0.354	0.157
O yolk (%)	0.736	0.396
Na yolk (%)	**0.032**	0.227
Mg yolk (%)	0.109	0.553
P yolk (%)	0.395	0.766
S yolk (%)	0.518	0.861
Cl yolk (%)	0.157	0.262
K yolk (%)	0.302	0.833
Ca yolk (%)	0.266	0.743
Se chorion (%)	0.400	0.849
C chorion (%)	0.762	0.854
O chorion (%)	0.763	0.347
Na chorion (%)	0.284	0.514
Mg chorion (%)	0.373	0.238
P chorion (%)	0.996	0.684
S chorion (%)	0.793	0.170
Cl chorion (%)	0.455	0.263
K chorion (%)	0.895	0.180
Ca chorion (%)	0.274	0.226
Lipid droplet distribution (ranked)	2.73 ± 0.90	1.98 ± 0.84

A.4 Discussion

Overall the results show that feeding a Sel-plex supplemented diet to broodstock brown trout has the potential to alter the physical structure and biochemical constituents of their eggs. Also, the linear regression of all parameters of egg quality on egg survival showed that there were both positive and negative linear relationships with novel (EDX analysis and chorion breaking strength) and pre-tested (fecundity) measures of egg quality. However, the validity of these results must be carefully considered due to the rearing environments significantly confounding influence on egg quality.

All significant results observed during this study were from one site only, Ae Fishery or S.C.E.N.E., and were not replicated in the opposite site. For example, significant differences in the lipid droplet distribution between fish fed different dietary treatments at Ae Fishery was not replicated at S.C.E.N.E.. This strongly indicates that environmental factors out with the remit of the current study affected broodstock and subsequently influenced the intrinsic properties of the egg itself. Environmental effects on egg quality are discussed in detail in Chapter 3.

A2 Effect of Individual variation on determinants of egg quality and egg survival in salmonids

A2.1 Introduction

Many factors are known to affect egg quality. A great deal of evidence exists regarding what are thought to be the major determinants of egg quality in fish. There are widespread reports that diet, endocrine status and the physiochemical conditions of the water, in which the fish are reared and the eggs they produce are incubated, are all variables which may affect egg quality (Bobe and Labbe, 2010; Brooks *et al.*, 1997). Individual variation is an important factor likely to affect not only fecundity but also egg quality. However, the effect of individual variation, influenced via genetic factors and behavioural mechanisms, has on egg quality is without a doubt one of the most poorly understood areas of finfish reproductive biology (Bobe and Labbe, 2010).

Studies have highlighted that differences in the type and abundance of maternal RNA within the eggs produced by female broodstock may cause significant differences in egg quality, and thus egg survival. For example, Stoddard *et al.*, (2005), found that early onset embryonic mortality of some rainbow trout eggs was caused by the insufficient transfer of important maternal genotypes to the egg during development. Furthermore, the social status of individual farmed fish, a function of their behaviour, is also likely to affect egg quality through the complex interactions between this type of conduct and physiological responses associated with reproduction (Bobe and Labbe, 2010). Despite the need for detailed analysis, information regarding how individual variation effects the constituents of the egg and how this effects egg quality is negligible (Brooks *et al.*, 1997).

A2.2 Materials and Methods

The details of how brown trout were held and how the eggs were processed can be found in Chapter 2 section 2.3, Chapter 3 section 3.3. and Appendix 1 section A.2.

The details of how Atlantic salmon were held and how the eggs were processed can be found in Chapter 4 section 4.3 and Chapter 5 section 5.3.

A2.2.1 Data analysis

All data presented as percentages were arcsine transformed before statistical analysis. The effect of individual variation on determinants of egg quality and egg survival was examined by one-way analysis of variance (ANOVA). Minitab® was used for data analysis.

A2.3 Results

A2.3.1 Variation in egg quality parameters between eggs from female brown trout

There was significant differences between brown trout held at Location 1 for non-activated breaking strength ($F_{[6,69]}$= 36.34, r^2= 0.78, p< 0.001), activated breaking strength ($F_{[6,69]}$= 80.00, r^2= 0.88, p<0.001), pre-activation egg diameter ($F_{[6,69]}$= 4.99, r^2= 0.32, p< 0.001), post activation egg diameter ($F_{[6,69]}$= 5.92, r^2= 0.36, p< 0.001), pre-activation egg weight ($F_{[6,69]}$= 59.44, r^2= 0.85, p<0.001), post activation egg weight ($F_{[6,69]}$= 47.82, r^2= 0.82, p< 0.001), yolk Se concentrations ($F_{[6,27]}$= 7.09, r^2= 0.67, p< 0.001) chorion Se concentrations ($F_{[6,25]}$= 4.51, r^2= 0.59, p=0.005) and egg survival ($F_{[6,13]}$= 19.39, r^2= 0.94, p< 0.001) (Table A2-1).

There was also significant differences between fish held at Location 2 for non-activated breaking strength ($F_{[9,99]}$= 5.36, r^2= 0.35, p< 0.001), activated breaking strength ($F_{[9,99]}$= 17.83, r^2= 0.64, p<0.001), pre-activation egg diameter ($F_{[9,94]}$= 7.92, r^2= 0.46, p< 0.001), post activation egg diameter ($F_{[9,94]}$= 2.63, r^2= 0.22, p= 0.010), pre-activation egg weight ($F_{[9,94]}$= 5.58, r^2= 0.37, p< 0.001), yolk Se concentrations ($F_{[9,39]}$= 2.43, r^2= 0.42, p< 0.033), Cl yolk concentration ($F_{[9,39]}$= 5.22, r^2= 0.61, p< 0.001), Ca yolk concentration ($F_{[9,39]}$= 2.83, r^2= 0.46, p< 0.016), chorion Se concentrations ($F_{[9,38]}$= 2.65, r^2= 0.45, p=0.022), C chorion concentration ($F_{[9,38]}$= 2.58, r^2= 0.44, p= 0.026), Mg chorion concentrations ($F_{[9,38]}$= 2.26, r^2= 0.41, p= 0.047), Cl chorion concentration ($F_{[9,38]}$= 2.73, r^2= 0.46, p= 0.019) and Ca chorion concentration ($F_{[9,38]}$= 3.68, r^2= 0.53, p= 0.004) (Table A2-1).

Table A2-1: Variation in determinants of egg quality and egg survival between brown trout held at both Location 1 and Location 2.

Parameters of egg quality	Location 1 (Mn ± Std dev)	Location 1 p-value	Location 2 (Mn ± Std dev)	Location 2 p-value
Non-activated breaking strength (N)	0.68 ± 0.67	**<0.001**	6.75 ± 1.61	**< 0.001**
Activated breaking strength (N)	1.38 ± 1.34	**<0.001**	20.22 ± 5.09	**< 0.001**
Pre-activation diameter (mm)	4.49 ± 0.32	**<0.001**	4.63 ± 0.44	**< 0.001**
Post activation diameter (mm)	5.00 ± 3.00	**<0.001**	5.16 ± 0.30	**0.010**
Pre-activation weight (g)	0.06 ± 0.01	**<0.001**	0.06 ± 0.01	**<0.001**
Post activation weight (g)	0.07 ± 0.01	**<0.001**	0.08 ± 0.09	0.261
Se yolk (%)	3.20 ± 2.71	**<0.001**	4.58 ± 1.43	**0.033**
C yolk (%)	51.01 ± 5.22	0.251	51.42 ± 5.13	0.296
O yolk (%)	33.19 ± 6.79	0.356	33.64 ± 6.51	0.243
Na yolk (%)	4.38 ± 1.29	0.118	4.45 ± 0.73	0.755
Mg yolk (%)	4.30 ± 1.35	0.349	3.98 ± 0.77	0.292
P yolk (%)	6.70 ± 3.59	0.551	5.68 ± 2.53	0.730
S yolk (%)	7.18 ± 3.68	0.625	5.85 ± 2.51	0.305
Cl yolk (%)	5.07 ± 3.01	0.085	4.60 ± 2.41	**< 0.001**
K yolk (%)	5.93 ± 4.16	0.405	5.01 ± 2.50	0.081
Ca yolk (%)	4.11 ± 2.00	0.676	3.41 ± 1.85	**0.016**
Se chorion (%)	2.15 ± 2.22	**0.005**	4.02 ± 1.31	**0.022**
C chorion (%)	48.69 ± 3.28	0.334	47.76 ± 3.90	**0.026**
O chorion (%)	38.51 ± 4.32	0.229	39.40 ± 3.80	0.305
Na chorion (%)	4.05 ± 1.71	0.372	4.42 ± 1.27	0.060
Mg chorion (%)	4.56 ± 6.01	0.741	3.65 ± 1.21	**0.047**
P chorion (%)	4.40 ± 2.99	0.153	3.44 ± 1.48	0.826
S chorion (%)	4.22 ± 2.93	0.129	3.70 ± 2.42	0.269
Cl chorion (%)	2.69 ± 2.42	0.577	2.97 ± 4.67	**0.019**
K chorion (%)	3.55 ± 2.53	0.475	1.85 ± 1.57	0.080
Ca chorion (%)	3.03 ± 2.67	0.868	2.58 ± 3.50	**0.004**
Egg survival (%)	14.18 ± 17.86	**<0.001**	72.14 ± 13.87	0.059

A2.3.2 Variation in chorion quality parameters between eggs from female Atlantic salmon

There was significant differences between Atlantic salmon for un-eyed chorion breaking strength ($F_{[15,159]}$= 4.15, r^2= 0.30, p< 0.001), eyed chorion breaking strength ($F_{[15,159]}$= 11.56, r^2= 0.55, p< 0.001), Ca chorion concentrations ($F_{[15,71]}$= 9.59, r^2= 0.72, p< 0.001), Mg Chorion concentrations ($F_{[15,71]}$= 2.38, r^2= 0.39, p= 0.010), P chorion concentrations ($F_{[15,71]}$= 4.82, r^2= 0.56, p<0.001), S chorion

concentrations ($F_{[15,71]}$= 3.32, r^2= 0.47, p= 0.001), and egg survival ($F_{[15,71]}$= 2.85, r^2= 0.43, p= 0.002) (Table A2-2).

Table A2-2: Variation in determinants of egg quality between Atlantic salmon from a single population.

Parameters of egg quality	Population (Mn ± Std dev)	Population p-value
Un-eyed chorion breaking strength (N)	1.47 ± 0.27	**<0.001**
Eyed chorion breaking strength (N)	1.73 ± 0.33	**<0.001**
Ca chorion (%)	4.78 ± 3.69	0.197
Fe chorion (%)	10.35 ± 11.59	**0.002**
K chorion (%)	4.93 ± 3.25	0.526
Mg chorion (%)	9.40 ± 4.12	**0.010**
N chorion (%)	54.56 ± 18.22	**<0.001**
P chorion (%)	11.65 ± 5.49	**<0.001**
S chorion (%)	10.77 ± 6.68	**0.001**
Se chorion (%)	4.17 ± 7.06	0.078
Zn chorion (%)	3.22 ± 4.97	0.281

A2.3.3 Intra-population variation in egg survival rates from eggs of female Atlantic salmon fed supplemented and non-supplemented diets.

The results show that there was significant variation in egg survival between Atlantic salmon fed a Se-supplemented diet and held in Tank 4 ($F_{[9,19]}$= 28.78, r2= 0.96, p< 0.001). There was no significant difference in egg survival rates between Atlantic salmon fed a non-supplemented diet (Table A2-3).

Table A2-3: Variation in egg survival between individual Atlantic salmon fed Se supplemented and non-supplemented diets

Tank and Treatment	Egg Survival (Mn ± Std dev) (%)	Egg Survival (p-value)
Tank 1 + Se supplemented diet	59.64 ± 14.85	0.065
Tank 2 + Se supplemented diet	60.50 ± 12.68	0.571
Tank 3 + Se supplemented diet	54.17 ± 12.20	0.519
Tank 4 + Se supplemented diet	51.65 ± 17.02	**<0.001**
Tank 5 + Non-supplemented diet	70.04 ± 9.22	0.108

A2.4 Discussion

Overall these results show that individual variation or 'family effects' has the potential to significantly alter the physical structure and biochemical

constituents of the egg. The effect of parental genetics on egg quality has, so far, remained poorly investigated. However, the results from the current study suggest that individual variation strongly influence determinants of egg quality and egg survival.

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